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Title of Dissertation: "Refining the Mechanisms of *Heniparvirus*-Mediated Membrane Fusion Through Mutagenesis of *Hendra virus* Envelope Glycoproteins"

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ABSTRACT

Refining the Mechanism of Henipavirus-Mediated Membrane Fusion through Mutagenesis of the Hendra virus envelope glycoproteins

Kimberly A. Bishop-Lilly, Ph.D. 2007

Thesis supervisor: Dr. Christopher C. Broder, Professor and Director, Emerging Infectious Diseases Graduate Program

Hendra virus (HeV) and Nipah virus (NiV) are two newly emergent zoonoses within the family *Paramyxoviridae* that are currently classified as Biological Safety Level 4 (BSL-4) agents because of their lethality and the lack of approved therapeutics or vaccines. Paramyxoviruses are enveloped viruses and their entry into cells is via Class I fusion. Although inferences can be made based on what is known about fusion and entry by other prototypical Class I fusion viruses, there remain steps in the process of fusion and entry by paramyxoviruses which are not well understood. For instance, although it is know that the fusion glycoprotein (F) and the attachment glycoprotein (G) interact to promote fusion, it is not known how, when, or through what domains they interact. In addition, although the henipavirus cellular receptors have recently been discovered to be ephrinB2 and ephrinB3, it is not known which residues of G bind the receptors or how exactly receptor binding by G triggers F-mediated fusion. Using site-directed mutagenesis of HeV G, we have identified residues in the putative β-sheets 1 and 4 of the globular head of G that are critical for binding both receptors. In addition, we have investigated the contribution of a series of stalk region isoleucines to the protein's various

functional characteristics, and found that mutation of these residues causes G to assume a post-receptor binding conformation in the absence of receptor– a subtle conformational alteration which blocks fusion. In addition, through characterization of these mutants, we have been able to draw certain conclusions regarding the mechanism of fusion and entry by henipaviruses. Specifically, our data support a model whereby F and G are pre-associated prior to receptor binding, and receptor-induced conformational changes in G serve to trigger F-mediated fusion. Furthermore, our data suggest a role for the stalk of a paramyxovirus attachment protein in maintenance of conformation and regulation of the timing of events in fusion. These findings lend insight into the mechanism of Class I fusion by paramyxoviruses and could lead to the design of specific viral inhibitors in the future.

REFINING THE MECHANISM OF HENIPAVIRUS-MEDIATED MEMBRANE FUSION THROUGH MUTAGENESIS OF THE HENDRA VIRUS ENVELOPE GLYCOPROTEINS

By

Kimberly Ann Bishop-Lilly

Dissertation submitted to the Faculty of the Emerging Infectious Diseases Program of the Uniformed Services University of the Health Sciences

F. Edward Hébert School of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy 2007

Preface

Portions of this manuscript have been published as: **Bishop, K.A., Stantchev, T.S., Hickey, A.C., Khetawat, D., Bossart, K.N., Krasnoperov, V., Gill, P., Feng, Y-R., Wang, L., Eaton, B.T., Wang, L-F., and C.C. Broder.** 2007. Identification of *Hendra virus* G glycoprotein residues that are critical for receptor binding. Journal of Virology **81:** 5893-901 or submitted as **Bishop, K.A., Hickey, A.C., Patch, J.R., Bossart, K.N., Wang, L-F., and C.C. Broder.** Residues in the stalk domain of the *Hendra virus* G glycoprotein modulate conformational changes associated with receptor binding.

The human ephrinB2/FC protein was cloned, characterized, and purified by Vasgene Therapeutics, Inc. The human ephrinB2/s-tag protein was cloned and characterized by myself and purified by Yan Ru Feng.

Dedication

I wish to thank the faculty of the Emerging Infectious Diseases Program and the staff of the Graduate Education Office of the Uniformed Services University of the Health Sciences for providing me this opportunity to further my education. I owe an especial thanks to Dr. Lee Metcalf and Dr. Christopher Broder, both of whom have helped me tremendously in various ways during my time here, supporting my endeavors and giving excellent guidance and support.

I would like to thank the other graduate students in the Broder lab, present and former, for their day-to-day help and laughter. Additionally, I am grateful to our collaborators at Vasgene Therapeutics and at CSIRO, including a former student of USUHS, Katharine Bossart.

I would also like to thank Trupti (Tinka) Brahmbhatt, with whom I have shared each step of this journey, from studying to qualifying and even now. She has been the best study partner, the best giver of pep talks, and my best friend. Without her, I might not have gotten to where I am now.

Last but not least, I would like to thank my husband, Joe, for being so patient and kind through all of this and for always believing in me….

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Chapter 1: Introduction

Bats as reservoirs of emerging viruses

An emerging disease is one which has either newly appeared in a population or, although it has existed in the past, has increased in incidence and/or expanded its geographic range. Currently, the incidence of emerging and re-emerging diseases is on the rise, and has been cited as a major transition in the relationship between humans and microbes (86). A disproportionate number of emerging diseases are caused by RNA viruses (40), and at least two thirds (40) to three quarters (80) of such newly emergent human diseases originate from animals.

Bats have been recently implicated as the suspected reservoir for many emerging viral diseases. Bats have long been known to serve as reservoirs for Rabies virus, and are now known to be reservoirs of the closely related Australian Bat Lyssavirus as well, of which the first recognized human death occurred in 1996 (reviewed in (52)). In addition, SARS-like coronaviruses (SL-CoVs)– closely related to severe acute respiratory syndrome coronavirus (SARS-CoV), the causative agent of SARS, which emerged in 2002 and 2003, have recently been identified in three species of Chinese horseshoe bats (genus *Rhinolophus*), and these bats are now thought to be the natural viral reservoir (47, 50). Bats are also hypothesized to be a potential reservoir of Ebola and Marburg viruses, and recent evidence has indicated that three species of fruit bats appear to be naturally and asymptomatically infected with Ebola virus in nature (34). Indeed, experimentally inoculated Wrinkle-lipped bats have been shown to support Ebola virus replication *in vivo* (77).

Interestingly, all the above-mentioned viruses have single-stranded ribonucleic acid (RNA) genomes of negative polarity as well as similar genome organizations and replication strategies, which place them in the Order *Mononegavirales* (44). In fact, the vast majority of viruses associated with bats are RNA viruses. The apparent predilection of RNA viruses for bats (or vice versa) is not understood, and very little is known about the immune systems of bats in general, but the above-mentioned observations suggest that there are aspects of the bat's immune response to RNA viruses that are worthy of further investigation (89).

Within the Order *Mononegavirales* also resides the Family *Paramyxoviridae* (43), which contains several other newly emergent viruses associated with bats, including Menangle virus, Tioman virus, Hendra virus, and Nipah virus.

Emergence of Hendra and Nipah viruses

Hendra virus emerged in 1994 in the state of Queensland, Australia, in two separate spillover events. The first recognized outbreak occurred in Hendra, a suburb of Brisbane, Australia in September. In this outbreak, 18 horses became ill with an apparent respiratory infection, and 14 of these died. Three additional horses seroconverted without evidence of illness. A horse trainer who had contact with the sick horses' secretions contracted the illness, presumably through abrasions on his hands and arms, and died soon after of cardiac arrhythmias brought about by respiratory illness. During this outbreak, a stable hand also became infected with Hendra virus, although his illness was less severe and he recovered (30).

A year later, it became evident that a third human had been infected with Hendra virus, actually a month prior to the first two recognized cases. This man, who lived on a

horse farm in Mackay, about 620 miles away, had assisted in the autopsy of two dead horses and then become ill with aseptic meningitis. Similar to the horse trainer described above, he is presumed to have contracted the virus through abrasions on his hands and forearms. He recovered from his illness but died one year later of relapsing encephalitis (66).

Serological testing, morphological analysis, and sequencing of isolated virus from the above cases identified the causative agent as a new member of the *Paramyxovirinae* subfamily, although this virus did not fit into any of the pre-existing genera. Originally termed Equine Morbillivirus, it was renamed Hendra virus (HeV). Serological surveys of wildlife identified flying foxes (or fruit bats) in the genus *Pteropus* as the probable reservoir for infection, and virus was later isolated from the bats for confirmation (59).

Subsequent to these events, in 1998 an outbreak of severe encephalitis of unknown etiology began in Malaysia and spread eventually into Singapore. The virus infected both pigs and humans, causing 105 of 265 human cases to die– a case fatality rate of approximately 40%. In order to control the outbreak, over 1 million pigs were culled (20), resulting in a large negative impact on the pig farming industry in Malaysia. Upon molecular characterization, this virus was found to be distinct from all other known viruses, but was most closely related to HeV. It was subsequently named Nipah virus (NiV) after a village in which it emerged (20). Again, fruit bats were found to be the reservoir of infection. (Serendipitously, it was during this search for the NiV reservoir that another novel paramyxovirus, Tioman virus, was identified in bats (21)). A new genus, *Henipavirus*, was proposed to house HeV and NiV (83), and they were both classified as Biological Safety Level 4 (BSL-4) agents.

Since their initial outbreaks, both HeV and NiV have continued to cause outbreaks. In 1999 (41), 2004 (38), and 2006, HeV sickened horses in Queensland, Australia. Then, in a second 2006 outbreak, HeV surfaced for the first time in the state of New South Wales, potentially widening its scope (2). Ironically, at the time of this manuscript's preparation, there appears to be a current outbreak of HeV in Queensland, AU, which has so far sickened two horses and one veterinarian (3). Similarly, NiV has may have widened its range as well, causing outbreaks in India in 2001 (18) and 2007 (72), and Bangladesh in 2001, 2003 (29), 2004 (4-6), and 2005 (51). In addition to potentially widening their geographic range, these viruses have taken on other frightening aspects. For instance, the most recent outbreaks of NiV have involved case-fatality rates of approximately 75% (4), higher incidence of Acute Respiratory Distress Syndrome (ARDS), and what appears to be human to human transmission in the absence of an amplifying animal host (6). These characteristics, along with the ease of obtaining the viruses from nature and the lack of any approved therapeutics or vaccine, make henipaviruses an important area of research for biodefense purposes.

Pathogenesis

HeV and NiV are unusual paramyxoviruses in that they are zoonotic and highly pathogenic (reviewed (27)). Henipaviruses have a broad host range and with the exception of fruit bats, it has been demonstrated that all infectable animal species can succumb to fatal henipavirus infection. In NiV infection of humans, death may occur as quickly as 10 days after onset of symptoms (reviewed (49)). Overall, the spectrum of disease caused by henipaviruses in humans ranges from seroconversion in the absence of symptoms, to myalgia, headache, and lethargy followed by respiratory disease and renal

failure, to Acute Respiratory Distress Syndrome (ARDS), to acute encephalitis. 20% of patients who recover from NiV encephalitis will exhibit residual neurological defects. In an interesting parallel with another paramyxovirus, Measles virus (MeV), approximately 10% of henipavirus cases may present with an initial infection that appears to be self-limiting and then present again, up to 4 years later, with relapsing encephalitis (28). How or whether this latter manifestation of disease is at all analogous to Subacute Sclerosing Panencephalitis (SSPE), a neurological disease which occurs years after infection with MeV, is unclear.

We and others have recently shown that HeV and NiV are capable of utilizing the same cellular receptors, ephrinB2 and ephrinB3 (7, 60). Identification of the viral receptors sheds some light on the pattern of pathogenesis seen in NiV infected humans and animals experimentally infected with HeV and NiV. As there have been less human cases of HeV than NiV, there is considerably more known about the pathogenesis of NiV in humans. *In vivo*, NiV has a tropism for neurons and endothelial cells, with viral antigen being found there as well as in smooth muscle and tunica media of small arteries (but not veins). Syncytia, or fused, multinucleated giant cells, are a hallmark of infection. Syncytia and viral antigen are found not only in endothelial tissues, but also in lymphoid tissue, including the white pulp of the spleen. EphrinB2, a molecule with tyrosine kinase activity, is involved in axonal guidance and angiogenesis. Not surprisingly, ephrinB2 is expressed in neurons, endothelial cells, white pulp of the spleen, and smooth muscle of arteries, but not veins. Thus the pattern of ephrinB2 expression in tissues essentially mirrors the pattern of NiV pathogenicity. Additionally, the gene encoding ephrinB2 has been found in every animal genome searched thus far and varies little amongst animal

species, which could explain the unusually broad host range of henipaviruses (reviewed (49)).

A major outcome of henipavirus infection is damage to organs such as the lung, heart, and kidneys, as well as the central nervous system (CNS). Antiviral antibody is evident in serum before it is found in cerebrospinal fluid (CSF). This finding, in conjunction with extensive lymphoid necrosis, leads to speculation that the virus may first replicate in lymphoid tissue and from there, establish viremia and infect the CNS (28, 49). The ability of henipaviruses to cause systemic infection is relatively unusual amongst paramyxoviruses, and may in part also be related to the widespread distribution of another host-expressed protein, Cathepsin-L. Cathepsin-L is the host protease responsible for cleavage of the henipavirus fusion protein precursor to a fusion-active form, meaning that without cleavage of the F glycoprotein by Cathepsin-L, the virus's ability to spread from cell to cell and create syncytia would be diminished (64). Many paramyxovirus F glycoproteins are cleaved by host proteases which have more limited expression, in some cases, localized to the respiratory tract. In contrast, the endosomal protease Cathepsin-L is expressed widely in the human body, and this additional feature may allow the virus to spread throughout the host unchecked (28).

In addition, another pathogenic determinant of henipaviruses is that they encode several proteins which have evolved to interfere with the host's innate response to viruses. This feature is common to paramyxoviruses in general, and is encoded through editing of the P gene into several different protein products. However, unlike other paramyxoviruses, the henipaviruses have a multi-pronged anti-interferon strategy that involves a unique function of the W gene product (which will be discussed further

below)(27, 28), and perhaps this additional anti-interferon strategy helps tip the balance in favor of virus over host, leading to uncontrolled systemic infection and death.

The genome and its encoded proteins

Paramyxovirus genomes consist of unsegmented, single-stranded, negative-sense RNA (44). The genomes of NiV and HeV are larger than most other members of the *Paramyxoviridae* family, which was one factor considered as criteria for separation into their own genus, *Henipavirus*. This increased genome length consists mostly of additional nucleotides in the 3' untranslated regions (82, 83). Like the other paramyxoviruses, the HeV and NiV genomes conform to the "rule of six," which means that the number of nucleotides is evenly divisible by six (37). This factor appears to be important for the way that the nucleocapsid (N) protein interacts with genomic RNA, with one nucleocapsid protein molecule interacting with every six nucleotides (44).

Although their genomes are larger, the relative gene order is conserved as compared to other paramyxoviruses, with the N gene being first, followed by the P (phosphoprotein) , M (matrix) , F (fusion) , G (attachment), and L (large/polymerase) genes (in a 3' to 5' order). The N, P, and L proteins form a complex which is responsible for replication of viral RNA; polymerase activity residues within the L protein itself. In addition to the full-length, unedited P gene product, the P gene encodes the V and W proteins through a transcriptional mechanism involving addition of untemplated G nucleotides, as well as the C protein, which is encoded by an alternative start codon within the P gene. The V protein functions in anti-interferon signaling, in a similar way as that of other paramyxoviruses. However, unlike other paramyxoviruses, the W protein exhibits an additional anti-interferon activity, targeting the interferon pathway in a

different subcellular compartment– the nucleus. The smaller C protein also exhibits anti-interferon activity *in vitro*, although whether it plays a role *in vivo* is unclear (27, 28). The matrix protein, which underlies the viral membrane, plays a key role in organization of viral proteins during the process of budding from the host cell (65). The F and G glycoproteins, which are expressed on the surface of the virion, are essential for virus binding and entry into permissive host cells (28, 44) (discussed below).

Membrane fusion mediated by paramyxovirus envelope glycoproteins

Like other paramyxoviruses, HeV and NiV express on their surface two envelope glycoprotein spikes– a trimeric fusion glycoprotein (F) and a tetrameric attachment glycoprotein. Unlike that of many other paramyxoviruses, the henipavirus attachment glycoprotein contains neither hemagglutinating nor neuraminidase activities, thus its designation G versus a hemagglutinin (H) or a hemagglutinin-neuraminidase (HN) glycoprotein. Like the vast majority of paramyxoviruses, both F and an attachment glycoprotein are required for fusion and entry. The G glycoprotein mediates attachment of the virion to the target cell, after which F mediates membrane fusion through a pH-independent, Class I fusion process. Class I fusion is a mechanism involving a trimeric viral glycoprotein which facilitates merger of viral and host cell membranes through insertion of a hydrophobic fusion peptide into the target membrane, followed by tightly controlled conformational changes. Prototypical Class I fusion glycoproteins include Influenza HA and HIV Env. Conversely, Class II fusion is the other means by which viral envelope glycoproteins mediate membrane fusion. In this process, the viral fusion glycoprotein exists as a dimer on the surface of the virion, which then becomes a trimer upon activation. Rather than a fusion peptide, Class II fusion proteins generally contain a

fusion loop which gets inserted into the target membrane (reviewed in (42)). General features of paramyxovirus fusion proteins will be further discussed below.

Paramyxoviruses are unlike some other viruses which utilize Class I fusion in that the receptor binding and fusion activities are housed in two separate glycoproteins- either F or G alone will not suffice. For the vast majority of paramyxoviruses, F and the attachment protein are required to work together in order for fusion to occur, but there is also an additional requirement– that the F glycoprotein and the attachment glycoprotein are derived from the same virus. In other words, the Measles virus F cannot function alongside HeV G in fusion. However, closely related viruses within the same genus can in some cases show heterotypic complementation and can mediate membrane fusion, such as HeV and NiV or MeV and CDV. This specificity of interaction applies not only to fusion activity but also generally to the ability of the fusion and attachment glycoproteins to coprecipitate (reviewed in (8)).

Receptor binding and other functions of the attachment glycoprotein

We and others have recently identified the receptor for HeV and NiV to be ephrinB2 (7, 60), a molecule which is expressed on endothelial and nervous tissues, essentially mirroring the pattern of pathogenesis in an infected patient (recently reviewed in (49)). Additionally, it has also been shown that ephrinB3 can be utilized as an alternate entry receptor by NiV (61). To date, however, it has not been clear which residues of henipavirus G are responsible for binding the receptors. The only other paramyxoviruses which are known to utilize host cell proteins as opposed to sialic acid moieties as receptors are Measles virus (MeV), Rinderpest virus, and Canine Distemper virus (CDV) (reviewed in (91, 92)). There is currently no solved structure for

henipavirus G, but based on sequence alignments with other paramyxovirus attachment proteins like MeV H and Newcastle disease virus (NDV) HN, along with computer-assisted modeling, the globular head domain of G is hypothesized to form what is called a "six-bladed beta propeller" motif atop a mostly alpha-helical stalk domain (87, 96).

Thus far, some of the various functions of paramyxovirus attachment glycoproteins have been mapped to broadly defined regions of the glycoprotein. The receptor binding activity of paramyxovirus attachment proteins is generally found to lie in the globular head domain (recently reviewed in (8)). The neuraminidase active site of HN proteins also lies in the globular head domain, overlapping with the receptor binding site in human Parainfluenza virus-3 (hPIV-3) HN (48) and with one of two potential receptor binding sites of NDV HN (13, 23, 97). In addition, we and others have found several important antigenic sites to be concentrated in the globular head of the molecule: for instance, eight sites of HeV G neutralization escape mutations are found in the globular head (87). However, elucidation of which domain of the attachment protein is directly responsible for F-engagement and/or F-specificity has been less clear, with various studies implicating either the stalk domain (26, 57, 75, 76, 79) or areas in both the stalk domain and the globular head domain (81). In addition, the stalk domain has been implicated not only in F-engagement and/or determining F-specificity, but also as the minimal domain required for dimerization in the case of H (68), as being a critical requirement for functional neuraminidase activity in the case of HN (25, 84), and interestingly, in maintenance of the antigenic structure of HN (25).

Within the stalk domain reside a series of hydrophobic isoleucine, leucine, and valine residues which are well conserved among the various members of the

Paramyxovirinae subfamily and have been hypothesized to form a heptad repeat (HR) structure similar to those found in the F glycoprotein. Previous work by Stone-Hulslander et al. (76) and by Wang et al. (84) found that mutation of these residues in the stalk region to alanine impaired Newcastle Disease virus (NDV) HN's fusion-promotion ability and neuraminidase activity, respectively. However, although these residues are conserved across the *Paramyxovirinae*, the contribution of these residues to the various functions of other paramyxovirus attachment proteins had not yet been explored.

The fusion glycoprotein

Unlike the attachment protein which is not proteolytically processed, the F glycoprotein is synthesized as F_0, a precursor that is proteolytically cleaved by cellular Cathepsin-L (64) into the F_1 and F_2 subunits, which are disulfide-linked. This cleavage event probably serves to "prime" F for fusion, as it is a prerequisite for fusion activity (63), but is not sufficient for "triggering" of F. Once F is triggered, it undergoes a dramatic refolding upon itself to insert its hydrophobic fusion peptide into the target cell membrane. F possesses two heptad repeat domains, HRA and HRB, which interact to promote its refolding. Upon triggering, HRA forms a coiled-coil structure, into the grooves of which pack the HRB regions. This structure is termed the "six helix bundle," and is thought to represent the "post-fusion" conformation of F. Recently, crystal structures of two paramyxovirus F proteins have been solved: one which is thought to represent the pre-fusion structure (95), and one which is thought to resemble the post-fusion structure (94). Analysis of these two crystal structures supports the idea that dramatic refolding of F occurs upon triggering and that the rearrangements resemble

those of other Class I viral fusion glycoproteins, such as that of Influenza virus HA and Human Immunodeficiency Virus (HIV) Env (73).

The presence of HRA and HRB domains is conserved across the family of *Paramyxoviridae* F glycoproteins, and it has been demonstrated that synthetic peptides derived from these regions can serve as potent inhibitors of fusion by binding to transiently exposed regions of F fusion intermediates and blocking transition to the post-fusion conformation (11, 12, 71, 90). Leucine zipper motifs contained inside these HR regions are critical to fusogenicity (14, 15). In addition, some paramyxovirus F glycoproteins have been found to possess a third heptad repeat region within the F_2 subunit, HRC (19, 46, 55, 67). According to a crystal structure of NDV F, this HRC region interacts directly with HRA (19) and is critical for proper folding and transport of F (67). It is not clear however, exactly what mechanistic role this HRC domain performs in fusion. Additionally, it is not clear whether or not there exist other similar HR-like domains in F which perform specific roles in fusion.

Models of paramyxovirus fusion

As for the vast majority of paramyxoviruses, coexpression of the attachment glycoprotein along with the fusion glycoprotein is required for functional fusion-promotion activity, and as F and G are known to interact (as evidenced by coprecipitation-based assays) it is generally thought that upon receptor binding to G, it then somehow triggers F-mediated fusion (recently reviewed in (8)). The mechanism by which this supposed cross talk between G and F occurs, however, is not presently understood.

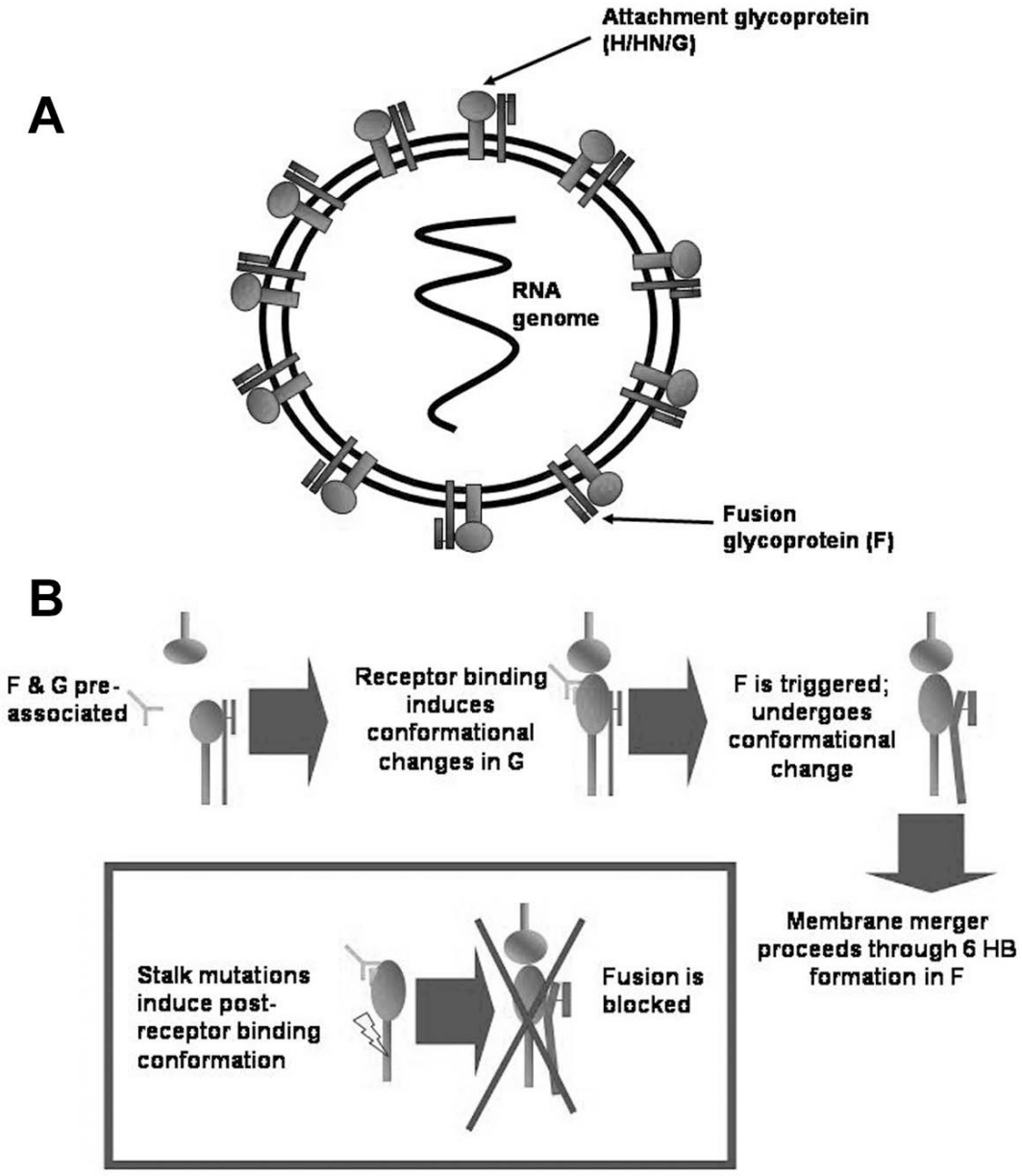
Although inferences can be made based on what is known about other viruses which mediate Class I fusion, there still remain many specific questions about the mechanism of membrane fusion mediated by paramyxoviruses. For instance, although F and G are known to interact, it is not known by what domains of either glycoprotein this interaction is mediated. In addition, the stoichiometry of the F/G interaction is not known. The nature of the "triggering" event for F is not entirely understood, nor are the details of all the conformational changes involved. Historically, there has also been debate concerning the timing of events, i.e. do F and G interact only after receptor binding or are they pre-associated prior to receptor binding? Further, if they are pre-associated prior to receptor binding, do they dissociate once G has bound receptor?

Currently, there exist in the literature two models for paramyxovirus fusion. The first model hypothesizes that receptor binding induces conformational changes in G and that these conformational changes in G somehow trigger F-mediated fusion. Data to support this model have been obtained for NDV HN. Furthermore, receptor-induced conformational changes have also been detected in other examples of Class I viral fusion, for example, in Avian Sarcoma/Leukosis Virus SU (24, 33) and the more well-studied HIV gp120 (31). Alternatively, a second model stipulates that rather than conformational changes, receptor binding induces a change in the oligomeric status of G, and that it is this change in oligomeric status which serves to trigger F-mediated fusion. Data to support this model have been obtained for hPIV-5 HN (both models were recently reviewed in (8)).

Data obtained in the current study using henipavirus envelope glycoproteins support a model whereby F and G are pre-associated on the virion or cell surface, as will

be described in Chapter 3, and receptor binding induces conformational changes in G which serve to "trigger" or activate F for fusion. These conformational changes in G are detectable through the use of monoclonal antibodies (mAbs) and appear to be regulated by an isoleucine-rich motif in the stalk domain of the G glycoprotein, as will be described in Chapter 5. (A schematic of the favored model is depicted in **Fig. 1**.) Additionally, specific isoleucine residues within a hydrophobic motif in the HeV F protein also appear to play an important role in mediating membrane fusion as well, and this will be discussed in Chapter 4.

Figure 1: Paramyxovirus envelope glycoproteins interact to mediate membrane fusion. A) Schematic of a typical paramyxovirus particle, illustrating two major envelope glycoproteins on its surface. The attachment glycoprotein is designated H, HN, or G depending on whether it possesses hemagglutinating, hemagglutinating and neuraminidase, or neither activity (HN, H, or G), and is responsible for attachment of the virion to a cellular receptor. The attachment glycoprotein interacts with a second glycoprotein, the fusion glycoprotein, which is designated F. The F glycoprotein is directly responsible for mediating fusion of the virion's membrane with that of the host cell. In B) is a schematic of the favored model of how the fusion process occurs. Prior to receptor binding, the fusion and attachment glycoproteins are associated on the virion or cell surface. Binding of G to the cellular receptor induces conformational change(s) in G (depicted arbitrarily by subtle vertical elongation of the head region) which are detectable in vitro through the use of monoclonal antibodies. These conformational changes serve to "trigger" F for fusion, and F undergoes conformational changes of its own that result in six-helix bundle formation (6HB) and membrane merger. Alternatively, certain mutations introduced into the stalk region of G can cause G to assume the post-receptor binding conformation in the absence of bound receptor. These premature conformational changes result in a block in fusion, suggesting an important role for the stalk domain of the attachment protein in regulating conformation and the timing of events in fusion.


A
Attachment glycoprotein (H/HN/G)
RNA genome
Fusion glycoprotein (F)
B
F & G pre-associated
Receptor binding induces conformational changes in G
F is triggered; undergoes conformational change
Membrane merger proceeds through 6 HB formation in F
Stalk mutations induce post-receptor binding conformation
Fusion is blocked

Aims and hypotheses

The overall goal of the current study was to utilize henipaviruses as a model system for studying membrane fusion mediated by paramyxoviruses. Through mutagenesis of henipavirus glycoproteins, I have sought to provide evidence that supports one model of fusion over the other. Specifically, the aims and hypotheses of this project were as follows:

Specific aim 1: Identify receptor binding residues in HeV G

- Hypothesis: The receptor binding site of G is conformation-dependent
- Hypothesis: The globular head domain mediates interaction of G with receptor, with at least some important elements located in β-sheets 5 and 6, based on homology with MeV H

Specific aim 2: Explore the contribution of a specific series of hydrophobic residues between HeV F HRA and HRB to henipavirus fusion

- Hypothesis: Conserved isoleucines between HRA and HRB are critical to HeV F-mediated membrane fusion

Specific aim 3: Investigate the contribution of an isoleucine-rich domain in the stalk region of HeV G to the protein's various functional characteristics and roles

- Hypothesis: Conserved isoleucine residues in the stalk domain of G are critical for fusion
- Hypothesis: Conserved isoleucine residues in the stalk domain of G are not critical for receptor binding, oligomerization of G, or interaction with F

Chapter 2: Materials and Methods

Cells and culture conditions

HeLa-USU cells and PCI-13 cells have been described previously (7, 10). 293T cells were obtained from Dr. G. Quinnan (Uniformed Services University, Bethesda, MD). HeLa-USU and 293T cells were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% cosmic calf serum (CCS) (HyClone, Logan, UT), and 2 mM L-glutamine (DMEM-10). PCI-13 cells were maintained in DMEM-10 plus 100 mM Hepes buffer. All cell cultures were maintained at 37°C in 5% CO_2.

Glycoprotein constructs and mutagenesis

Conversion of specific residues of HeV G or HeV F to alanine or glutamine was performed via site-directed mutagenesis using the QuickChange II Site-directed Mutagenesis Kit (Stratagene, Cedar Creek, TX). The template for the reactions consisted of a C-terminal myc epitope-tagged version of HeV G or an untagged version of HeV F, each in the vaccinia virus-based plasmid pMCO2 (16). In most cases, oligonucleotide primers were approximately 43 bases long, and annealing temperature used in polymerase-chain-reaction (PCR) was 56.0°C. A list of primers is presented in **Tables 1-4**. Those reactions which did not work well at 56.0°C were repeated using a gradient of annealing temperatures in order to determine the ideal conditions. All mutation-containing constructs were sequence verified.

Table 1. **Names and sequences of oligonucleotides used to delete potential N-linked glycosylation sites from stalk mutant of HeV G**

Mutation	Oligo name	Oligo sequence (5' to 3')
		AAA TTG GTA AGC CTG AAC AAT CAA CTA TCT GGT
N13Q	ACHG-1F	AAA ATC AAG GAT
		ATC CTT GAT TTT ACC AGA TAG TTG ATT GTT CAG
	ACHG-1R	GCT TAC CAA TTT
		ATG AAT ATC ATG ATA ATT CAA CAA TAC ACC AGA
N72Q	ACHG-2F	ACG ACT GAT AAT
		ATT ATC AGT CGT TCT GGT GTA TTG TTG AAT TAT
	ACHG-2R	CAT GAT ATT CAT
		CCT TTA AAG ATT CAT GAG TGT CAA ATC TCT TGT
N159Q	ACHG-3F	CCG AAT CCT TTG
		CAA AGG ATT CGG ACA AGA GAT TTG ACA CTC ATG
	ACHG-3R	AAT CTT TAA AGG
		CAT GTG GGA GAT CCT ATC CTT CAA AGT ACT TCC
N306Q	ACHG-4F	TGG ACA GAG TCA
		TGA CTC TGT CCA GGA AGT ACT TTG AAG GAT AGG
	ACHG-4R	ATC TCC CAC ATG
		CCA AGG ACC GAA TTT CAA TAT CAA GAC TCT AAT
N378Q	ACHG-5F	TGT CCC ATA ATT
		AAT TAT GGG ACA ATT AGA GTC TTG ATA TTG AAA
	ACHG-5R	TTC GGT CCT TGG
		AGA TCA GGA CTA TTG AAG TAT CAA CTA TCT CTT
N417Q	ACHG-6F	GGA GGA GAC ATC
		GAT GTC TCC TCC AAG AGA TAG TTG ATA CTT CAA
	ACHG-6R	TAG TCC TGA TCT
		CCT CTA AGA GTA CAG TGG AGA CAA AAC AGT GTG
N481Q	ACHG-7F	ATT TCT AGA CCT
		AGG TCT AGA AAT CAC ACT GTT TTG TCT CCA CTG
	ACHG-7R	TAC TCT TAG AGG
		GCT GGT GTT TAT TTA AAC AGT CAA CAA ACT GCA
N529Q	ACHG-8F	GAG AAC CCT GTG
		CAC AGG GTT CTC TGC AGT TTG TTG ACT GTT TAA
	ACHG-8R	ATA AAC ACC AGC

Table 2. **Names and sequences of oligonucleotides used for site-directed mutagenesis in globular head of HeV G**

Mutation	Oligo name	Oligo sequence (5' to 3')
D254A	254S	CAA AGG ATA ATA GGG GTG GGT GCG GTA TTG GAT AGG GGT GAT AAG
	254AS	CTT ATC ACC CCT ATC CAA TAC CGC ACC CAC CCC TAT TAT CCT TTG
D257A	257S	GGG GTG GGT GAG GTA TTG GCT AGG GGT GAT AAG GTG CCA TC
	257AS	GAT GGC ACC TTA TCA CCC CTA GCC AAT ACC TCA CCC ACC CC
D260A	260S	GTG AGG TAT TGG ATA GGG GTG CTA AGG TGC CAT CAA TGT TTA TG
	260AS	CAT AAA CAT TGA TGG CAC CTT AGC ACC CCT ATC CAA TAC CTC AC
K261A	261S	GAG GTA TTG GAT AGG GGT GAT GCG GTG CCA TCA ATG TTT ATG ACC
	261AS	GGT CAT AAA CAT TGA TGG CAC CGC ATC ACC CCT ATC CAA TAC CTC
D326A	326S2	CTT GCT GTA AGA CCA AAA AGT GCT AGT GGA GAC TAC AAT CAG
	326AS2	CTG ATT GTA GTC TCC ACT AGC ACT TTT TGG TCT TAC AGC AAG
D329A	329S	GCT GTA AGA CCA AAA AGT GAT AGT GGA GCC TAC AAT CAG AAA TAC ATC GC
	329AS	GCG ATG TAT TTC TGA TTG TAG GCT CCA CTA TCA CTT TTT GGT CTT ACA GC
G439A	439S	GCT GAC AAT AGA TTG ACC ATC GCT TCT CCT AGT AAG ATA TAC
	439AS	GTA TAT CTT ACT AGG AGA AGC GAT GGT CAA TCT ATT GTC AGC
K443A	443S	GAC CAT CGG TTC TCC TAG TGC GAT ATA CAA TTC CCT AGG TCA AC
	443AS	GTT GAC CTA GGG AAT TGT ATA TCG CAC TAG GAG AAC CGA TGG TC
N446A	446S	GGT TCT CCT AGT AAG ATA TAC GCT TCC CTA GGT CAA CCC GTT TTC
	446AS	GAA AAC GGG TTG ACC TAG GGA AGC GTA TAT CTT ACT AGG AGA ACC
G449A	449S	GTA AGA TAT ACA ATT CCC TAG CTC AAC CCG TTT TCT ACC AGG C
	449AS	GCC TGG TAG AAA ACG GGT TGA GCT AGG GAA TTG TAT ATC TTA C
K465A	465S	CAT ATT CTT GGG ATA CGA TGA TTG CAT TAG GCG ATG TTG ATA CCG TTG
	465AS	CAA CGG TAT CAA CAT CGC CTA ATG CAA TCA TCG TAT CCC AAG AAT ATG
D468A	468S	GAT ACG ATG ATT AAA TTA GGC GCT GTT GAT ACC GTT GAC CCT C
	468AS	GAG GGT CAA CGG TAT CAA CAG CGC CTA ATT TAA TCA TCG TAT C
D470A	470S	CGA TGA TTA AAT TAG GCG ATG TTG CTA CCG TTG ACC CTC TAA GAG TAC
	470AS	GTA CTC TTA GAG GGT CAA CGG TAG CAA CAT CGC CTA ATT TAA TCA TCG
E553,D554A	5534S	CCT TTA CCA AGT TCC ACT GGC TGC AGC TGA CAC AAA TGC ACA AAA AAC C
	5534AS	GGT TTT TTG TGC ATT TGT GTC AGC TGC AGC CAG TGG AAC TTG GTA AAG G
K560A	560S	GAA GAT GAC ACA AAT GCA CAA GCA ACC ATC ACA GAT TGC TTC TTG C
	560AS	GCA AGA AGC AAT CTG TGA TGG TTG CTT GTG CAT TTG

		TGT CAT CTT C
D564A	654S	CAA ATG CAC AAA AAA CCA TCA CAG CTT GCT TCT TGC TGG AGA ATG TC
	564AS	GAC ATT CTC CAG CAA GAA GCA AGC TGT GAT GGT TTT TTG TGC ATT TG
E569A	569S	CAT CAC AGA TTG CTT CTT GCT GGC GAA TGT CAT ATG GTG TAT ATC AC
	569AS	GTG ATA TAC ACC ATA TGA CAT TCG CCA GCA AGA AGC AAT CTG TGA TG

Table 3. **Names and sequences of oligonucleotides used for site-directed mutagenesis in stalk region of HeV G**

Mutation	Oligo name	Oligo sequence (5' to 3')
I83A	R83S	CTG ATA ATC AGG CAC TAG CCA AAG AGT CAC TCC AGA GTG
	R83AS	CAC TCT GGA GTG ACT CTT TGG CTA GTG CCT GAT TAT CAG
I94A	HG94S	CCA GAG TGT ACA GCA ACA AGC CAA AGC TTT AAC AGA CAA AAT C
	HG94AS	G ATT TTG TCT GTT AAA GCT TTG GCT TGT TGC TGT ACA CTC TGG
I101A	R101S	CAA AGC TTT AAC AGA CAA AGC CGG GAC AGA GAT AGG CCC C
	R101AS	GGG GCC TAT CTC TGT CCC GGC TTT GTC TGT TAA AGC TTT G
I105A	HG105S	CAG ACA AAA TCG GGA CAG AGG CAG GCC CCA AAG TCT CAC TAA TTG
	HG105AS	CAA TTA GTG AGA CTT TGG GGC CTG CCT CTG TCC CGA TTT TGT CTG
I112A	HG112S	GAT AGG CCC CAA AGT CTC ACT AGC TGA CAC ATC CCG CAC CAT C
	HG112AS	G ATG GTG CGG GAT GTG TCA GCT AGT GAG ACT TTG GGG CCT ATC
I120A	HG120S	GAC ACA TCC CGC ACC ATC ACA GCT CCT GCT AAC ATA GGG TTA C
	HG120AS	G TAA CCC TAT GTT AGC AGG AGC TGT GAT GGT GCG GGA TGT GTC
I124A	HG124S	CCA TCA CAA TTC CTG CTA ACG CAG GGT TAC TGG GAT CCA AG
	HG124AS	CTT GGA TCC CAG TAA CCC TGC GTT AGC AGG AAT TGT GAT GG
I131A	HG131S	GGG TTA CTG GGA TCC AAG GCA AGT CAG TCT ACC AGC AG
	HG131AS	CTG CTG GTA GAC TGA CTT GCC TTG GAT CCC AGT AAC CC
I138A	HG138S	GAT AAG TCA GTC TAC CAG CAG TGC TAA TGA GAA TGT TAA CG
	HG138AS	CGT TAA CAT TCT CAT TAG CAC TGC TGG TAG ACT GAC TTA TC
I155A	HG155S	CTC TTC CTC CTT TAA AGG CTC ATG AGT GTA ATA TCT CTT G
	HG155AS	C AAG AGA TAT TAC ACT CAT GAG CCT TTA AAG GAG GAA GAG
I160A	G160S	GAT TCA TGA GTG TAA TGC CTC TTG TCC GAA TCC TTT GCC
	G160AS	GGC AAA GGA TTC GGA CAA GAG GCA TTA CAC TCA TGA ATC
I174A	HG174S	CTT TCA GAG AAT ACC GAC CAG CCT CAC AAG GGG TGA GTG ATC
	HG174AS	GAT CAC TCA CCC CTT GTG AGG CTG GTC GGT ATT CTC TGA AAG

Table 4. **Names and sequences of oligonucleotides used for site-directed mutagenesis of HeV F**

Mutation	Oligo name	Oligo sequence (5' to 3')
		CCG AGG ACT TCG ACG GCC TTG CAG AAA GTG ATA GCA TAA
L257A	F257S	CAG G
		CCT GTT ATG CTA TCA CTT TCT GCA AGG CCG TCG AAG TCC
	F257AS	TCG G
		GCC TTT TAG AAA GTG ATA GCG CAA CAG GCC AGA TAG
I262A	F262S	TCT ATG TAG
		CTA CAT AGA CTA TCT GGC CTG TTG CGC TAT CAC TTT CTA
	F262AS	AAA GGC
		GTG ATA GCA TAA CAG GCC AGG CAG TCT ATG TAG ATC TCA
I266A	F266S	GTA G
		CTA CTG AGA TCT ACA TAG ACT GCC TGG CCT GTT ATG CTA
	F266AS	TCA C
		GAT AGC ATA ACA GGC CAG ATA GCC TAT GTA GAT CTC AGT
V267A	F267S	AGC
		GCT ACT GAG ATC TAC ATA GGC TAT CTG GCC TGT TAT GCT
	F267AS	ATC
V269A	F269S	CAG GCC AGA TAG TCT ATG CAG ATC TCA GTA GCT ATT AC
	F269AS	GTA ATA GCT ACT GAG ATC TGC ATA GAC TAT CTG GCC TG
		CAG ATA GTC TAT GTA GAT GCC AGT AGC TAT TAC ATA
L271A	R271S	ATA GTA AGG
		CCT TAC TAT TAT GTA ATA GCT ACT GGC ATC TAC ATA
	R271AS	GAC TAT CTG
		GTA GAT CTC AGT AGC TAT TAC GCA ATA GTA AGG GTG
I276A	F276S	TAT TTT CC
		GGA AAA TAC ACC CTT ACT ATT GCG TAA TAG CTA CTG
	F276AS	AGA TCT AC
		GAT CTC AGT AGC TAT TAC ATA GCA GTA AGG GTG TAT
I277A	F277S	TTT CCC
		GGG AAA ATA CAC CCT TAC TGC TAT GTA ATA GCT ACT
	F277AS	GAG ATC
		CTC AGT AGC TAT TAC ATA ATA GCA AGG GTG TAT TTT
V278A	F278S	CCC ATA C
		GTA TGG GAA AAT ACA CCC TTG CTA TTA TGT AAT AGC
	F278AS	TAC TGA G
		GCT ATT ACA TAA TAG TAA GGG CGT ATT TTC CCA TAC
V280A	F280S	TAA CAG
		CTG TTA GTA TGG GAA AAT ACG CCC TTA CTA TTA TGT
	F280AS	AAT AGC
		GTA AGG GTG TAT TTT CCC GCA CTA ACA GAG ATC CAA
I284A	F284S	CAG GC
		GCC TGT TGG ATC TCT GTT AGT GCG GGA AAA TAC ACC
	F284AS	CTT AC
		GGG TGT ATT TTC CCA TAG CAA CAG AGA TCC AAC AGG
L285A	F285S	CTT ATG
		CAT AAG CCT GTT GGA TCT CTG TTG CTA TGG GAA AAT
	F285AS	ACA CCC
		GTA GAT CTC AGT AGC TAT TAC GCA GCA GTA AGG GTG
I276A*	276S2	TAT TTT CC
		GGA AAA TAC ACC CTT ACT GCT GCG TAA TAG CTA CTG
	276AS2	AGA TCT AC
		GCC TTT TAG AAA GTG ATA GCG CAA CAG GCC AGG CAG
I262A**	R262S	TCT ATG
		CAT AGA CTG CCT GGC CTG TTG CGC TAT CAC TTT CTA
	R262AS	AAA GGC

(*) indicates this mutation was made in I262A background

(**) indicates this mutation was made in I266, I277, I284A background

Amino acid sequence alignments

The following amino acid sequences were obtained from Pubmed and utilized in alignments: SeV HN accession number AB005795, CDV HN accession numbers AF014953, L13194, and L13195, Mumps HN accession number AB040874, MeV H accession number AB016162, hPIV-3 HN accession number AB012132, hPIV-2 HN accession number X57559, hPIV-1 HN accession number AF457102, and NDV HN accession number AF309418, along with the amino acid sequences for our HeV and NiV G clones (AAC831932 and AF212302, respectively). Global protein alignments were created in Clone Manager Software (Scientific and Educational Software, Cary, NC) using a Blosum 62 scoring matrix.

Ephrin constructs

A soluble, secreted, S-peptide-tagged version of human ephrinB2 was generated from a full-length cDNA (accession number NM004093) (Origene, Rockville, MD) by PCR. The 5' primer included an external SalI site, a Kozak sequence and ephrinB2-specific sequence. The 3' primer included ephrinB2 specific sequence, the codons for the S-peptide tag (KETAAAKFERQHMDS), a stop codon and an external SalI site. The PCR generated a SalI-flanked product that encoded the first 233 amino acids of ephrinB2 fused in frame to the S-peptide tag. The PCR product was gel purified and cloned into TOPO (Invitrogen Corp., Carlsbad, CA), and subsequently subcloned into pMCO2. The final construct was sequenced and protein expression was verified in cell lysates and supernatants by immunoprecipitation using the epitope tag and western blotting with ephrinB2-specific polyclonal and monoclonal antibodies. Recombinant vaccinia virus vKAB8 (human ephrinB2/s-tag) was generated and used to infect BSC-1 cells in roller

bottles at an MOI of 5. Supernatant was collected from the roller bottles and clarified by centrifugation. EphrinB2 protein was purified via an S-bead column and eluted with 0.2 M sodium citrate, pH 2, with immediate neutralization by 1 M Hepes, pH 8. Binding of purified protein to Henipavirus G and S-agarose was verified and the protein was aliquoted and stored at -80°C.

A second human ephrinB2/FC construct was generated by PCR using primers which generated a 695 bp fragment of the ephrinB2 extracellular domain (B2EC) and subcloned into pEF6-TOPO-myc vector (Invitrogen). The resulting pEF6-TOPO-myc-B2EC plasmid was digested with HindIII-BamHI and a 687 bp fragment was subcloned into pCXFc vector, which is a pcDNA3.1/Zeo(+) (Invitrogen) vector containing the sequence of human Fc between BamHI and XbaI sites. The human ephrinB2/FC protein was produced in HEK-293 cells as a secreted protein by transfection of the pCXFc-B2EC with Lipofectamine 2000 (Invitrogen). Human ephrinB2/FC was purified from culture media by affinity chromatography using Protein-A agarose. After sample loading, Protein-A matrix was washed with PBS and absorbed ephrinB2/FC was eluted by 0.1M Glycin-HCl, pH 2.3 with immediate neutralization by 1M Tris-HCl, pH 8.5. The eluted protein was dialyzed against 50 mM Tris-HCl, 150 mM NaCl pH 8 buffer, binding reactivity of the ephrinB2/FC to EphB4 was verified, and protein was aliquoted and kept at -80°C.

Murine ephrinB2/FC, human ephrinB1/FC, and human ephrinB3/FC were obtained from R & D Systems, Minneapolis, MN.

Metabolic labeling and immunoprecipitations

Subconfluent HeLa-USU cells were transfected with plasmids encoding the various alanine mutation-containing envelope glycoproteins or wild-type envelope glycoprotein using the Fugene-6 transfection reagent (Roche, Indianapolis, IN). Cells were transfected with 3 μg total DNA per T-25cm^2 flask overnight followed by infection with wild-type vaccinia virus (strain WR) at a multiplicity of infection (MOI) of 10. At six hrs post-infection, the cells were washed and incubated overnight with methionine and cysteine-free minimum essential media (Invitrogen) containing 2.5% dialyzed fetal calf serum (Invitrogen), L-glutamine, and 100 μCi per ml of [35S]methionine-cysteine (Promix; Amersham Pharmacia Biotech, Piscataway, NJ). Approximately 16 hrs later, the cells were chased with complete media for 2 hrs, and cell lysates were prepared using lysis buffer (100mM Tris-HCl (pH 8.0), 100mM NaCl, and 1% Triton X-100), clarified by centrifugation and analyzed by immunoprecipitation and SDS-PAGE.

For coprecipitations of G with receptor, G-containing cell lysates were incubated with 3 μg human ephrinB3/FC (R & D Systems, Minneapolis, MN) or human EFNB2/s-tag followed by precipitation with either Protein-G Sepharose (Amersham) or S agarose (EMD Biosciences Inc, Madison, WI), respectively. For immunoprecipitations with G- or F-specific antibodies, 4 μl of a polyclonal antisera, 3 μg purified MAb or 5 μl concentrated hybridoma supernatant were incubated with envelope glycoprotein-containing lysate at 4°C for one hr or overnight. Samples were washed twice with lysis buffer followed by one wash with 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% sodium deoxycholate, and 0.1% SDS (DOC wash buffer). Samples were boiled in

sample buffer with 2-mercaptoethanol, analyzed by SDS-PAGE, visualized by autoradiography, and quantified by densitometry.

For coprecipitation assays of G with F glycoproteins, the F and G encoding plasmids were cotransfected into HeLa-USU cells. Cells were then infected and 16-18 hrs later, cell lysates were prepared as described above. Equivalent amounts of each lysate were pre-cleared with Protein-G Sepharose for 45 minutes at room temperature and then incubated at 4°C overnight with 5 µl rabbit polyclonal F_1-specific antisera or 5 µl rabbit polyclonal G-specific antisera, and then precipitated with Protein-G Sepharose, washed and boiled with 2-mercaptoethanol as described above, before being analyzed by SDS-PAGE and western blotting under reducing conditions with mouse polyclonal G-specific antisera at 1:20,000.

Immunoprecipitated proteins were quantified by spot-densitometry using AlphaEase Fc Software (Alpha Innotech, San Leandro, CA).

Monoclonal antibodies

Human monoclonal antibodies m101, m102.4, m106, and m106.3 were provided by Zhongyu Zhu, Protein Interactions Group, CCRNP, CCR, NCI-Frederick, NIH, Frederick, Maryland and BRP, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD in conjunction with Dimiter S. Dimitrov, Protein Interactions Group, CCRNP, CCR, NCI-Frederick, NIH, Frederick, Maryland. Mouse monoclonals 8H4 and H2.1 were provided by John R. White, CSIRO Division of Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria, AU. Mouse monoclonals hAH1.3, hAH2.1, hAH5.1, nAH22.4, nAH23.4, and nAH24.4 were provided by Andrew Hickey, Uniformed Services University, Bethesda, MD.

Conformational change assay

WT HeV G expressed in HeLa-USU cells as described above was pre-incubated for 1 hr at 37°C on a rotator with either 3 μg s-tagged human ephrinB2 or an equal amount of PBS. Following this pre-incubation, the lysate was subjected to immunoprecipitation with each available human or mouse monoclonal antibody for 1 hr at 4°C, followed by precipitation with Protein G and either immunoblotting or autoradiography.

Cell fusion assays

Fusion between F and G glycoprotein-expressing effector cells and permissive target cells was measured by a reporter gene assay in which the cytoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cytoplasm of the other contained the *E. coli lacZ* gene linked to the T7 promoter; β-galactosidase (β-Gal) is synthesized only in fused cells (9, 62). In a typical assay, plasmids encoding HeV F and each alanine mutant of G or no DNA (control/mock transfection) were transfected into HeLa-USU cells and allowed to express overnight as described above. 293T cells or PCI-13 cells served as receptor positive target cells. Vaccinia virus-encoded proteins were produced by infecting cells at a MOI of 10 and incubating infected cells at 31°C overnight. Cell fusion reactions were conducted with the various cell mixtures in 96-well plates at 37°C or at 42°C. The ratio of envelope glycoprotein-expressing cells to target cells was 1:1 (2×10^5 total cells per well, 0.2-ml total volume). Cytosine arabinoside (40 μg/ml) was added to the fusion reaction mixture to reduce nonspecific β-Gal production (9). For quantitative analyses, Nonidet P-40 Alternative was added (0.5% final) at 2.5 or 3.0 hrs and aliquots of the lysates were assayed for β-Gal at ambient temperature with the

substrate chlorophenol red-D-galactopyranoside (CPRG; Roche Diagnostics Corp.). Assays were performed in duplicate and fusion results were calculated and expressed as rates of β-Gal activity (change in O.D. at 570 nm per minute x 1,000) (62) in an MRX microplate reader (Dynatech Laboratories, Chantilly, VA).

Cell surface expression and normalization of cell fusion reactions

Various effector cell populations coexpressing HeV F along with either mutant or WT HeV G were prepared as described above for the reporter gene cell fusion assay. Prior to mixing the various effector populations with target cells, aliquots were made of each effector cell population (1×10^6 cells each). These aliquots were washed once with PBS to remove DMEM and then incubated with FITC-conjugated polyclonal antisera (Cell Signaling Technology, Inc, Danvers, MA) specific for the myc epitope tag on each G glycoprotein, in PBS with 3% goat serum on ice for 1 hr. Samples were washed three times before fixing in paraformaldehyde, 1.6% at 4°C, and then analyzed on a Beckman Coulter Epics XL flow cytometer.

The reporter gene assay was conducted as described earlier for each mutant or WT effector cell population and then the raw β-Gal readings were normalized to compensate for the difference between each mutant and the WT G glycoprotein's surface expression as measured by the anti-myc staining described above. These numbers were then divided by that of WT and multiplied by 100 to obtain the predicted % of WT fusion activity that each mutant would be expected to demonstrate when a mutant G glycoprotein would be expressed on the surface of effector cells at equivalent levels to WT. Most of the mutants (11 of 17) were found to be surface-expressed between 50% and 120% of that of WT G. Four mutants were found to be surface-expressed between

20 and 50% of WT G, and two mutants, K443A and E254A, were surface-expressed ~10% of WT. Cell surface expression levels of each G glycoprotein mutant construct were also analyzed by flow cytometry for some experiments using several mAbs, including a FITC-conjugated anti-myc mAb (Invitrogen) as well as by cell-surface biotinylation and probing assays and these additional analyses yielded similar results as compared to the anti-myc staining and flow-cytometry analysis. In addition, a subset of mutants and WT G was tested for surface expression in the presence and absence of HeV F coexpression using the polyclonal and monoclonal anti-myc antibodies in order to determine whether any variations in surface expression levels were evident. We found no differences in the cell surface expression levels of WT or any mutant G glycoprotein whether or not they were coexpressed with the F glycoprotein.

Cell surface biotinylation

HeLa-USU cells were transfected with the various envelope glycoprotein-encoding plasmids and allowed to express overnight as described above. Cells were washed 3 times with ice cold PBS and then cell surface proteins were biotinylated using 0.25 mg/ml EZ-Link NHS-Biotin (Pierce, Rockford, IL) in PBS for 30 min at 4°C. Following surface biotinylation, the cells were washed 3 times with ice cold PBS and cell lysates were prepared as described above. One half of each lysate was incubated with 100 µl 20% Agarose-Avidin D (Vector Laboratories, Inc., Burlingame, CA) in IP buffer (0.14 M NaCl, 0.1 M Tris, and 0.1% Triton) at 4°C overnight. Samples were then washed twice with lysis buffer followed by one wash with DOC buffer as described above. Precipitated proteins were then boiled in reducing sample buffer and analyzed by 4-20% Tris-Glycine gradient gel electrophoresis (Invitrogen), followed by transfer to

nitrocellulose and probing with G-specific mouse polyclonal antisera or F-specific rabbit polyclonal antisera at a concentration of 1:20,000.

Sucrose gradient density ultracentrifugation

Oligomeric characteristics of the various G alanine mutants were assessed essentially as described before (10). In short, the various alanine mutants or WT HeV G were expressed in HeLa-USU cells, which were metabolically labeled, chased, and lysed as described above. One half each lysate was layered onto a continuous 5-20% sucrose gradient and centrifuged 40,000 rpm for 20 hr at 4ºC, and then each gradient was fractionated into fractions of about 1-1.2 ml. Fractions were immunoprecipitated with 2 µl G-specific rabbit polyclonal antisera overnight at 4ºC and then analyzed by SDS-PAGE on 4-20% Tris-Glycine gels under reducing and non-reducing conditions.

Deglycosylation assay

G alanine mutants or WT HeV G were expressed in HeLa-USU cells and lysates prepared as described above. G-containing lysates were incubated overnight at 4ºC with 5 µl G-specific rabbit polyclonal antiserum**,** precipitated with 100 µl Protein-G Sepharose**,** and washed as described above. The G beads were then boiled 10 min in 45 µl 1X Glycoprotein Denaturing Buffer (New England Biolabs, Beverly, MA), centrifuged at maximum speed 4 min, and the supernatant removed to new tubes in which the deglycosylation reaction was conducted using 3 µl PNGase F (New England Biolabs) for either 0, 10, or 60 min at 37ºC. The reactions were mixed with reducing sample buffer, analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with G-specific mouse polyclonal antisera at 1:10,000.

Chapter 3: Identification of residues in the Hendra virus G glycoprotein critical for receptor binding

Introduction

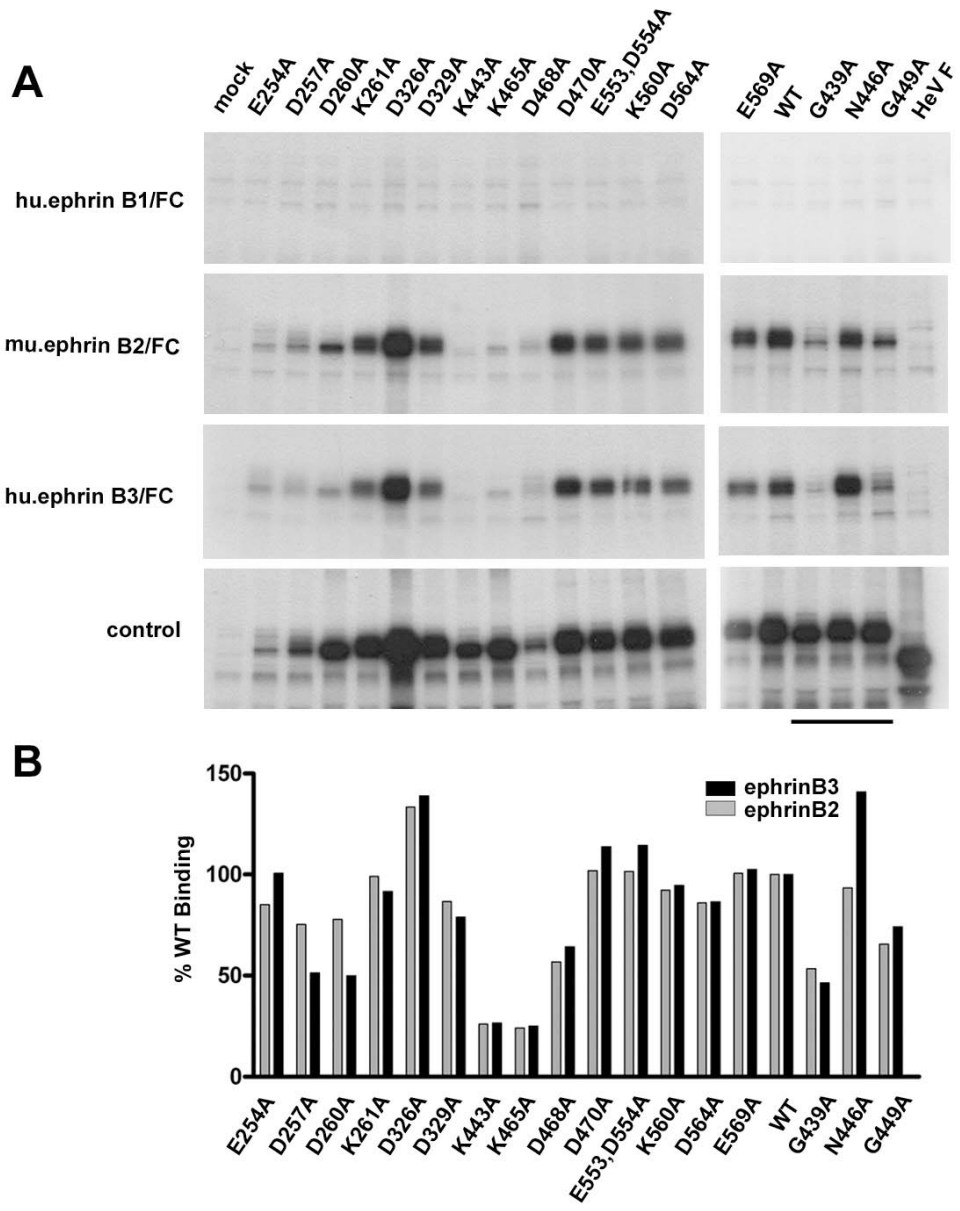
The first step in paramyxoviral entry into host cells is attachment of the virion to the target cell's surface via its receptor. The receptor for Hendra virus was recently identified to be ephrinB2 (7, 60). A crystal structure has not yet been solved for henipavirus G and, prior to the current study, it was not known what residues of G are utilized for binding ephrinB2. In this study, an alanine-scanning mutagenesis approach was employed, making use of receptor binding domain information about related viruses and predicted models of G and Measles virus attachment glycoprotein (H).

Results

Expression and receptor binding characteristics of HeV G glycoprotein mutants. In order to identify residues within the G glycoprotein that are important for engaging the ephrinB2 and ephrinB3 receptors an alanine-scanning mutagenesis strategy targeting specific charged amino acids in the predicted globular head domain of the molecule was conducted. First targeted was a series of charged amino acid residues within G which were predicted to correspond to homologous regions within the MeV H attachment glycoprotein known to be involved in its protein receptor interaction. The initial panel of G glycoprotein mutants consisted of 13 single amino acid point mutations and one double mutation, covering a range of charged residues in what is predicted to be β-sheets 1, 4, 6, and 2 loops- the β3/ β4 loop and the β5/ β6 loop (locations based on proposed model by Yu et al. (96)).

Previously, we had demonstrated a detectable interaction between soluble ephrinB2 with either HeV or NiV G using a coprecipitation assay (7). Here, this assay was employed to examine the panel of HeV G mutants as a preliminary screening method for G and ephrinB2 or ephrinB3 binding. As the extracellular domains of the murine and human ephrinB2 share 97% sequence identity (17), a commercially-available recombinant murine ephrinB2/FC protein was used for the initial screening of the mutants. After a 1.5 hour chase, metabolically-labeled cell lysates of the various alanine-substituted mutants or wild-type HeV G were coprecipitated with either the murine ephrinB2/FC or human ephrinB3/FC protein and the results are shown in **Fig. 2**. Since neither HeV or NiV G can bind and coprecipitate ephrinB1, a commercially available human ephrinB1/FC protein was used as a negative control for the specificity of the G glycoprotein-receptor interaction. In addition, since the receptor interaction is specific for the G glycoprotein of HeV, the HeV F protein was also used to demonstrate the specificity of binding as a further negative control. In parallel, identical amounts of lysates were also precipitated directly with rabbit polyclonal antisera against HeV G to control for expression levels of the various mutant glycoproteins. The precipitated proteins were washed and analyzed by SDS-PAGE and autoradiography. As demonstrated by the control immunoprecipitations, most of the 14 HeV G mutants were expressed to reasonably similar levels in comparison to WT G with the exception of the E254A mutant which was notably poorly expressed (**Fig. 2** bottom row). Notably, two G glycoprotein mutants, K443A and K465A, demonstrated

Figure 2. Expression and receptor binding of HeV G mutants. A: Plasmids encoding alanine substitution mutants of HeV G were transfected into HeLa-USU cells and metabolically labeled overnight. After a 1.5 hr chase, cell lysates were prepared and equivalent amounts of each lysate were immunoprecipitated with a HeV G-specific rabbit polyclonal antiserum (bottom row), or FC-tagged human ephrinB1, murine ephrinB2, or human ephrinB3. The HeV F protein was also subjected to the same conditions and serves as negative control for specificity of protein binding. In the case of the HeV F protein, F-specific antiserum was used as control (bottom row). The precipitated, metabolically labeled proteins were resolved by SDS-PAGE under reducing conditions and detected by autoradiography. Black line underneath lanes containing G439A, N446A, and G449A indicates that they are mutants which were made as a second panel to further explore the beta-sheet four region. B: The percent of wild-type ephrinB2 or B3 binding activity for each HeV G mutant is shown. Results were calculated using values obtained from densitometric measurements of autorad bands in comparison to the values obtained for WT HeV G.

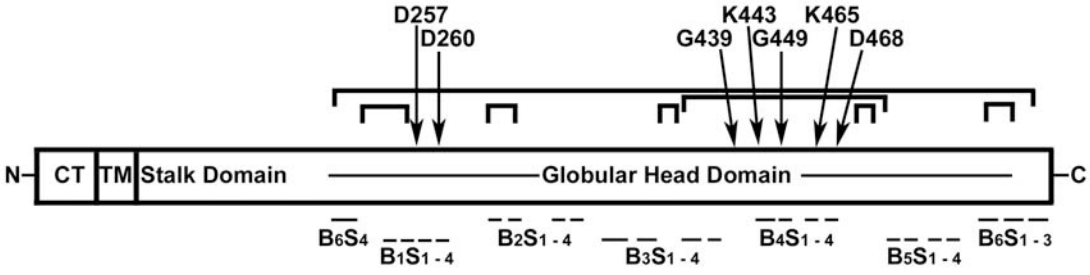

A
mock
E254A
D257A
D260A
K261A
D326A
D329A
K443A
K465A
D468A
D470A
E553,D554A
K560A
D564A
E569A
WT
G439A
N446A
G449A
HeV F
hu.ephrin B1/FC
mu.ephrin B2/FC
hu.ephrin B3/FC
control
B
% WT Binding
150
100
50
0
ephrinB3
ephrinB2
E254A
D257A
D260A
K261A
D326A
D329A
K443A
K465A
D468A
D470A
E553,D554A
K560A
D564A
E569A
WT
G439A
N446A
G449A

the most defective binding to both ephrinB2 and ephrinB3, with both mutants having approximately a 75% reduction in binding as compared to WT. In addition, several other mutants, D257A, D260A, and D468A, appeared to possess moderately impaired abilities to bind ephrinB2 and ephrinB3 as well, although these reductions in binding were not as dramatic as those seen for K443A and K465A. As expected, none of the mutants or WT G bound and coprecipitated with ephrinB1, and the HeV F protein precipitated only with F-specific antisera.

Using a predicted structure of HeV G (96) as a guide, residues K443, K465, and D468 all likely reside in the fourth beta sheet of the globular head region as illustrated in **Fig. 3**. Because K443A and K465A demonstrated such substantially impaired receptor binding capacity, several additional mutations in G within this general region were constructed. The analysis of the additional G glycoprotein mutants revealed two further mutations (G439A and G449A) that possessed moderately impaired ephrinB2 and ephrinB3 binding phenotypes (**Fig. 2**) and hence further delineated an important domain within G for receptor interaction, although neither mutation completely abrogated receptor binding.

Verification with human ephrinB2. The next step was to confirm that these HeV G mutations which impaired murine ephrinB2 binding also abrogated human ephrinB2 binding. In these studies, two human ephrinB2-derived constructs, an S-peptide tagged version and an FC-tagged version were employed. The mutants D257A, D260A, G439A, K443A, G449A, K465A, and D468A, as well as WT G, were expressed in HeLa-USU monolayers and the resulting lysates were subjected to coprecipitation with the two tagged forms of human ephrinB2, followed by western blotting with G-specific-

Figure 3. Schematic model of the G glycoprotein. A schematic model of the HeV G glycoprotein based on the model of Yu et al. (96) is shown. Putative disulfide bonds are represented as bridges above the schematic and β-sheets 1-6 of the globular head domain are depicted beneath the schematic.


D257
D260
K443
K465
G439
G449
D468
N
CT
TM
Stalk Domain
Globular Head Domain
C
B6S4
B1S1 - 4
B2S1 - 4
B3S1 - 4
B4S1 - 4
B5S1 - 4
B6S1 - 3

antisera. Using two alternative methods of coprecipitation, the patterns of binding of HeV and NiV G to either of these two human ephrinB2 constructs were remarkably similar to those obtained with the murine ephrinB2 protein (**Fig. 4** and **Fig. 2** respectively). For the sake of simplicity, only the data obtained with the S-peptide tagged version is shown. Specifically, the K465A and K443A mutants of HeV G were the most significantly impaired in binding human ephrinB2, with percent reductions in binding that were over 75%. In addition, the D257A, D260A, G439A, G449A, and D468A mutants also showed reduced binding activity which correlated very well to the results obtained using murine ephrinB2.

Characterization of HeV G glycoprotein mutants. Although it seemed unlikely that a single amino acid alteration could result in major conformational alterations in the G glycoprotein, the profound deficiencies in receptor binding demonstrated by several of the mutants, such as K443A and K465A, merited some additional examination of their overall structural and functional integrity. For these purposes, available conformation-dependent mAbs specific for the G glycoprotein were assessed for their abilities to bind the G glycoprotein mutants, and the results are summarized in **Table 5**. The mutants D260A, K443A, G449A, and K465A were all examined using six conformation-dependent mAbs to at least five distinct epitopes, including two mAbs previously shown to block receptor binding and thought to bind overlapping epitopes on or near the receptor binding site of G (98), and another mAb which is thought to bind G at a region analogous to the SLAM-binding site of MeV H (87). D260A and G449A were recognized by all six mAbs, although in some cases to a lesser extent than WT. K443A and K465A were recognized by three out of six mAbs. The

Figure 4. Binding of HeV G substitution mutants to human ephrinB2. A: Mutants defective in murine ephrinB2 and human ephrinB3 binding were expressed in HeLa-USU cells and lysates were precipitated with either soluble, S-epitope tagged human ephrinB2 plus S-agarose beads (top row) or polyclonal G-specific antiserum plus Protein-G beads (bottom row) as control. B: The percent of wild-type human ephrinB2 binding activity for each HeV G mutant is shown. Results were calculated using values obtained from densitometric measurements of autorad bands in comparison to the values obtained for WT HeV G.

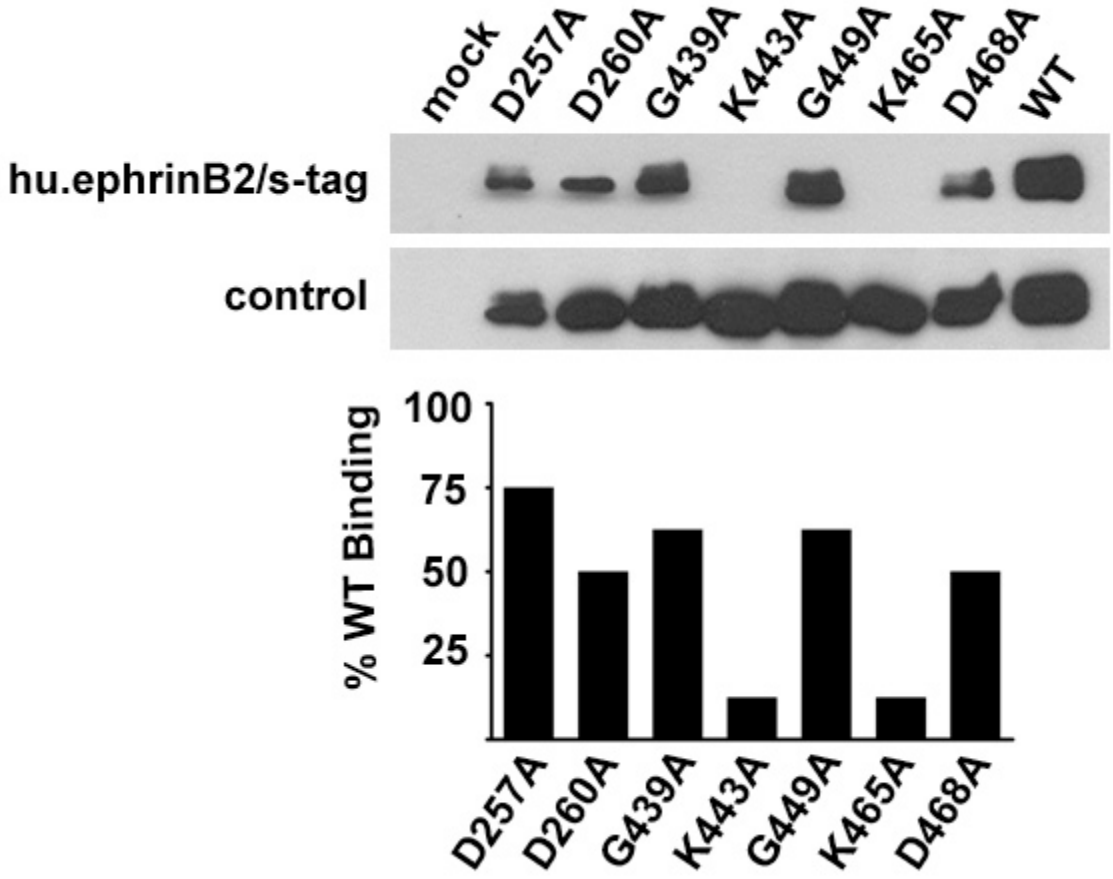

mock
D257A
D260A
G439A
K443A
G449A
K465A
D468A
WT
hu.ephrinB2/s-tag
control
% WT Binding
100
75
50
25
D257A
D260A
G439A
K443A
G449A
K465A
D468A

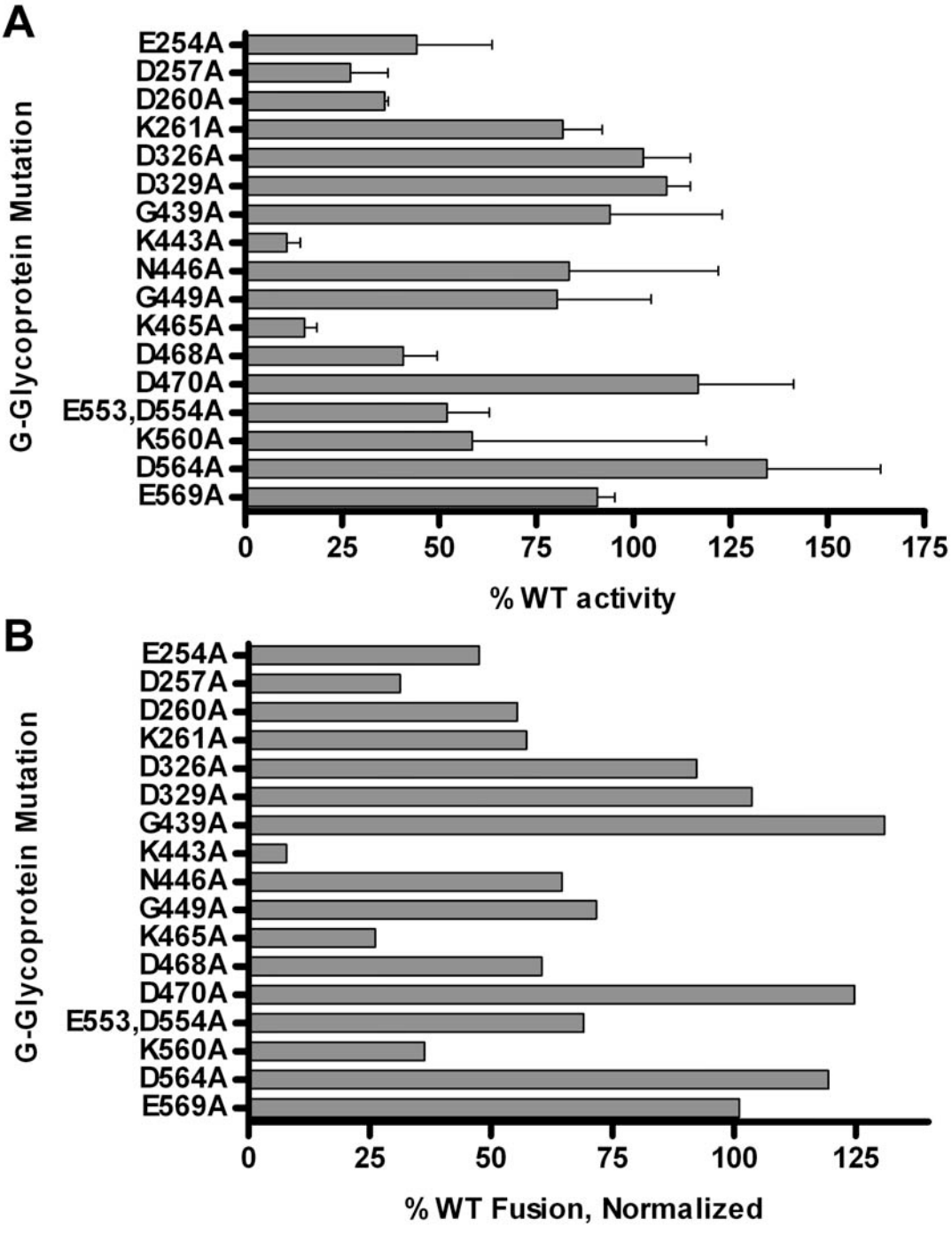
Table 5. Monoclonal antibody binding reactivities to HeV G glycoprotein mutants. Receptor binding-defective mutants of HeV G were tested for their reactivities to available conformation-dependent monoclonal antibodies by radio-immunoprecipitation, SDS-PAGE and autoradiography as described in the Methods. MAbs marked with an asterisk (*) are those thought to bind on or near the receptor binding domain. Percent reactivity of each G glycoprotein mutant is scored relative to WT G, with +++ as ≥ 90%, ++ as 40-90%, + as 30% or less reactivity, and – as non-detectable reactivity. NT = not tested.

	Conformation-dependent mAb					
G mutant	**hAH5.1**	**8H4**	**H2.1***	**102.4***	**101***	**106.3**
D257A	+++	+++	**NT**	+++	+++	+++
D260A	+++	+++	+	++	++	++
G439A	+++	+++	**NT**	+	+	++
K443A	+++	+++	-	-	-	+++
G449A	+++	+++	+	++	++	+++
K465A	+++	+++	-	-	-	+++
D468A	+++	+++	**NT**	+++	+++	+++

three mAbs that are implicated in binding to G on or near the receptor binding site, 101, 102.4, and H2.1, did not bind K443A and K465A, and exhibited reduced binding to D260A and G449A. The mutants D257A, G439A, and D468A were each recognized by five of five mAbs employed although the G439A mutant exhibited less reactivity with 101 and 102.4 in comparison to WT G. These binding data provide several important types of information. First, all of the mutants identified with impaired receptor binding are recognized by at least 3 or more conformation-dependent mAbs, providing strong evidence that they are not globally disrupted in structure. Second, they further confirm the importance of several amino acids residues in receptor binding because their alteration not only disrupts ephrinB2 and ephrinB3 interaction but also results in the loss of recognition by neutralizing mAbs known to bind to the G glycoprotein and compete with receptor binding (42).

Fusion-promotion activity of HeV G glycoprotein mutants. The next question to ask was whether the decreased receptor binding capacity of the various HeV G mutants correlated with a decrease in the glycoprotein's fusion-promotion activity when coexpressed with the F glycoprotein. Each of the HeV G mutant constructs was cotransfected with HeV F into an effector cell population and then tested for its ability to promote membrane fusion with a receptor-bearing partner cell population and these results are shown in **Fig. 5A**. The HeV G mutants K443A and K465A demonstrated the most profound defect in cell-fusion, with levels of 10.8 and 15.3 % of the level of fusion measured with wild-type HeV G respectively, when 293T cells served as receptor-positive partner cells. The overall deficiencies measured in cell-fusion promotion activity by the various mutants were qualitatively consistent with the receptor binding data, in

Figure 5. Cell fusion promotion activities of HeV G mutants. The alanine substitution mutants of HeV G were tested for their ability to promote cell fusion when coexpressed with HeV F using a quantitative, reporter gene, cell fusion assay. In A, the data shown is the average percentage of WT fusion levels measured for each mutant calculated from n=2 or 3 separate experiments, using 293T cells as the target population. Bars represent the range from multiple experiments. In B, aliquots of each effector cell population coexpressing HeV F along with the various mutants or WT G were subjected to flow cytometric analysis using a FITC-conjugated antibody specific for the myc epitope tag on G. Relative expression levels of the various mutants compared to WT G were then used to normalize the β-Gal readings from this reporter gene assay. The data are presented here as the predicted percent of WT activity each mutant would demonstrate if each mutant were expressed on the surface of cells to the same extent as WT. Each mutant effector population was tested for cell surface expression and fusion with several target cell lines on at least two separate occasions; a representative experiment with 293T target cells is shown.


A
G-Glycoprotein Mutation
E254A
D257A
D260A
K261A
D326A
D329A
G439A
K443A
N446A
G449A
K465A
D468A
D470A
E553,D554A
K560A
D564A
E569A
0
25
50
75
100
125
150
175
% WT activity
B
G-Glycoprotein Mutation
E254A
D257A
D260A
K261A
D326A
D329A
G439A
K443A
N446A
G449A
K465A
D468A
D470A
E553,D554A
K560A
D564A
E569A
0
25
50
75
100
125
% WT Fusion, Normalized

that mutants with defects in receptor binding (**Fig. 2**) were the same as those exhibiting defects in fusion promotion activity (**Fig. 5A**). In addition, the mutants D257A, D260A, and D468A, which were all identified as having moderate reductions in receptor binding ability (**Fig. 2**) also showed moderate reductions in fusion promotion ability. Two G mutants that exhibited only moderate receptor binding defects, G439A and G449A, did not possess significant fusion-promoting defects. The remaining HeV G mutants showed variable effects in fusion-promoting activities and are summarized in **Table 6** along with their locations within the predicted G structure. It was also noted that some G mutants possessed a hyperfusion-promoting phenotype, such as D564A, which exhibited an average fusion promotion ability of 34% more than WT (**Fig. 5A**).

As an additional means of assessing the conformational integrity and function of the mutant G glycoprotein panel, each was also assessed for its relative cell surface expression level in comparison to WT G. Each G glycoprotein mutant construct or WT G was expressed in at least two independent experiments and cell-surface expression was measured by flow cytometry using antibodies directed against a common myc epitope tag within each construct, as well as by cell-surface biotinylation assays as detailed in the Methods. All the G glycoprotein mutants were found to be expressed on the surface of cells at levels similar to or slightly less than that of WT G with the exception of E254A and K443A, each of which were expressed ~10% of WT G. Further, we also conducted these cell-surface expression assays in both the presence and absence of the partner glycoprotein, F, and it was further determined that F coexpression did not affect or influence the relative cell-surface expression levels of the various mutants in comparison to WT G.

Table 6. Predicted locations and fusion-promotion activities of G glycoprotein mutants. The putative locations of the G substitution mutants are listed based on a proposed structure (87, 96), along with their average fusion promotion activities. Receptor-positive 293T cells were employed as the target cell population. The percent of WT fusion-promotion activity for each mutant was calculated and the results of n=2 or 3 (as indicated in parentheses) independent experiments averaged.

G mutants and predicted locations		Percent of WT fusion
E254A	β_1S_3	44.2 (n=2)
D257A	β_1S_3	27.1 (n=2)
D260A	β_1L_{34}	35.9 (n=2)
K261A	β_1L_{34}	81.8 (n=2)
D326A	β_2S_4	102.6 (n=2)
D329A	β_2S_4	108.5 (n=2)
G439A	β_3/β_4Loop	94.0 (n=2)
K443A	β_4S_1	10.8 (n=2)
N446A	β_4S_1	83.5 (n=2)
G449A	β_4L_{12}	80.4 (n=3)
K465A	β_4S_3	15.3 (n=3)
D468A	β_4S_3	40.8 (n=3)
D470A	β_4S_3	116.7 (n=3)
E553, D554A	β_5/β_6Loop	52.1 (n=3)
K560A	β_5/β_6Loop	58.6 (n=3)
D564A	β_6S_1	134.3 (n=3)
E569A	β_6S_1	90.8 (n=3)

The cell fusion assay is dependent not only on the intrinsic fusion-promotion activities of the individual glycoproteins but also on the relative expression levels of each glycoprotein on the surface of cells. For this reason and because in this assay each G glycoprotein construct or WT G was expressed by transient transfection (the efficiency of which can vary from mutant to mutant), the cell-fusion data was normalized to account for any significant variations in cell-surface expression. Therefore, each mutant G or WT G glycoprotein was coexpressed with F in an individual effector cell population, as described above, for use in the reporter gene cell-fusion assay and also simultaneously assayed for G glycoprotein cell-surface expression levels in parallel in the same population of cells.

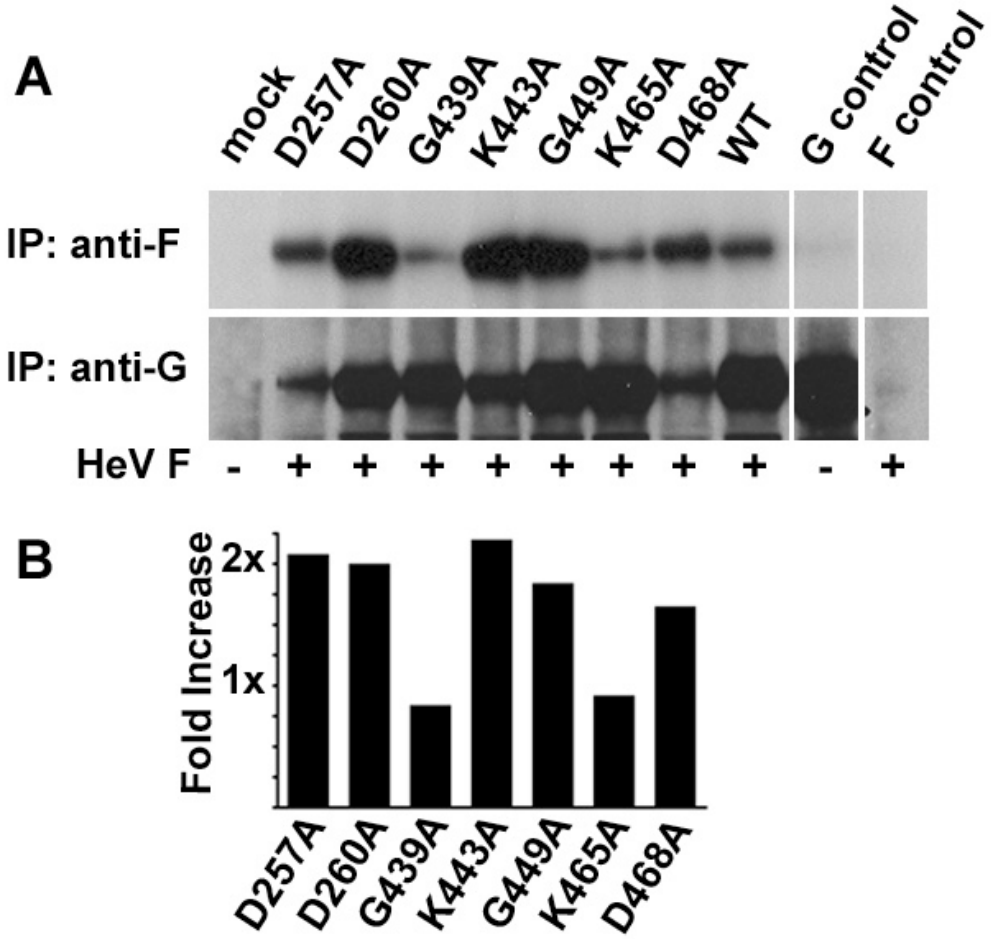
Following preparation of the various effector cell populations but prior to mixing with target cells, an aliquot of 1 x 10^6 cells was taken from each effector cell population, washed in PBS to remove residual cell culture media, and then stained with FITC-conjugated anti-myc antibody, for analysis by flow cytometry. The cell-fusion assay was then carried out on the same populations of effector cells. The reporter gene cell-fusion data were then later normalized as a means to account for any significant differences in cell-surface expression levels using the cell-surface expression data derived from the flow cytometry analysis as detailed in the Methods. The normalized cell fusion results are shown in **Fig. 5B.** The mutants D257A, D260A, K443A, G449A, K465A, and D468A, all of which were found to exhibit decreased binding to receptor (**Fig. 2** and **Fig. 4),** all possessed a decreased ability to promote fusion with a permissive target cell population following fusion data normalization to account for cell-surface expression level variations (**Fig. 5B**). The mutant D564A, which was noted to possess a

hyperfusion-promoting activity in previous fusion assays (**Fig. 5A**), retained this phenotype following normalization of the data; thus its phenotype is likely not a result of G glycoprotein over-expression on the cell surface. The mechanism(s) which underlie these apparent hyperfusion-promotion phenotypes is unclear. Interestingly, two additional mutants (D470A and G439A) also appear to have increased fusion-promoting phenotypes in comparison to WT (**Fig. 5B**) even though the G439A mutation confers a measurable decrease in the ability of G to bind receptor (**Fig. 2** and **Fig. 4**).

There were also three mutants, E254A, K261A, and K560A, that exhibited slightly decreased fusion-promotion ability after normalization (**Fig. 5B**) without significant receptor binding defects (**Fig. 2**).

F-interaction ability of G glycoprotein mutants. In addition to measuring the reactivity of the G glycoprotein mutants to conformation-dependent mAbs in immunoprecipitations and on the surface of cells, their ability to associate with their partner F glycoprotein was also assessed as a measure of the mutants' conformational integrity. The strength of the interaction between an F and its partner attachment glycoprotein can vary depending on the viral system being examined, but a biochemical interaction has been demonstrated with several paramyxoviruses (53, 56, 70, 93). The G glycoprotein mutants D257A, D260A, G439A, K443A, G449A, K465A, D468A, and wild-type HeV G were cotransfected with HeV F and analyzed by coprecipitation with a polyclonal antiserum raised against F followed by western blotting with a polyclonal antiserum raised against G (**Fig. 6A**, top row). The lysates were also immunoprecipitated with G-specific antiserum followed by blotting with a second G-specific antiserum in

Figure 6. Interaction of HeV G substitution mutants with HeV F. A: HeV G substitution mutants were coexpressed with HeV F in HeLa-USU cells. Lysates were immunoprecipitated with rabbit polyclonal F-specific antiserum and then blotted with mouse polyclonal G-specific antiserum as a test for the ability of the various G mutants to interact and coprecipitate with HeV F (top row), or immunoprecipitated with rabbit polyclonal G-specific antiserum and then probed with mouse polyclonal G-specific antiserum as a control for the relative expression level of each mutant (bottom row). HeV F (F control) and WT HeV G (G control) were also expressed singly in the absence of the partner glycoprotein and subjected to the same immunoprecipitation and blotting conditions to illustrate the specificity of the coprecipitation interaction. B: The relative F-binding ability of each HeV G mutant is shown in comparison to that of WT HeV G. Results were calculated using values obtained from densitometric measurements of autorad bands in comparison to the values obtained for WT HeV G.


A
mock
D257A
D260A
G439A
K443A
G449A
K465A
D468A
WT
G control
F control
IP: anti-F
IP: anti-G
HeV F - + + + + + + + + - +
B
Fold Increase
2x
1x
D257A
D260A
G439A
K443A
G449A
K465A
D468A

order to control for differing expression levels of the various mutants (**Fig. 6A**, bottom row). Both HeV F (F control) and HeV G (G control) were expressed singly in the absence of the partner glycoprotein to illustrate the specificity of the coprecipitation reaction. Under these conditions, each of the mutants retained the ability to bind and coprecipitate with F. In fact, when quantified by densitometry (**Fig. 6B**), mutants D257A, D260A, K443A, G449A, and D468A all demonstrated an approximately two-fold increased amount of G bound to F in comparison to the WT G.

Discussion

Using a site-directed mutagenesis strategy, conformation-dependent receptor binding elements in the predicted globular head region of the HeV G envelope glycoprotein have been identified. The regions identified here as important for receptor engagement consist of residues located in several strands of putative beta sheet four as well as two residues residing in beta sheet one. The data are complementary to the findings of Guillaume et al (36), who identified putative receptor binding residues in NiV G residing in the globular head region in areas similar to those found to be critical for MeV H receptor binding, although no biochemical or immunological characterizations were made with those mutants glycoproteins. In addition, the data are also consistent with earlier findings of Negrete et al. (60), where a deletion mutant of NiV G composed of residues 437-464 (Δ437-474 NiV G) had lost an ability to bind to the surface of receptor-positive 293T cells. According to a proposed structural model of HeV G (87, 96) residues 437-464 lie within β_4, and in the current study, five residues located within this region were identified important for receptor binding, namely G439, K443, G449, K465, and D468, which are crucial to HeV G's ability to bind ephrinB2 or ephrinB3.

Thus, it is likely that the binding defect of the Δ437-474 NiV G construct is due to the absence of amino acid residues G439, G449, K443, K465, and D468, all of which were found to be important for receptor binding in the present study.

In addition, differences observed in mAb reactivity of the various G glycoprotein mutants also correlated with their receptor binding phenotypes. Of the six mAbs, three are predicted to bind G at or near the domain(s) of the glycoprotein responsible for receptor binding. Accordingly, it was these three mAbs which showed the most striking difference in reactivity of the receptor binding defective mutants as compared to WT G. Specifically, H2.1, 101, and 102.4 showed a considerable decrease in their ability to recognize the mutants G439A, K443A, and K465A, as well as smaller but still noticeable effects in their ability to recognize D260A and G449A (**Table 5**). The correlation between loss of ephrinB2 and ephrinB3 binding ability and the loss of reactivity to mAbs 101 and 102.4, which were previously shown to block receptor binding (42), supports the conclusion that domains of G identified in this study are indeed likely involved in receptor binding. In addition, the correlation between losses in receptor binding ability and the decrease in reactivity to H2.1 by these same mutants further supports the hypothesis that HeV G and MeV H may have similarities in the location of their respective receptor binding sites, as this antibody is thought to bind G in regions analogous to the SLAM-binding domain of MeV H (38).

Interestingly, it was also demonstrated that all of the mutants which were defective in receptor binding were still able to interact with and coprecipitate with their partner glycoprotein, HeV F (**Fig. 6**). Surprisingly, the majority of these mutants which demonstrated decreased receptor binding were found to exhibit an apparent increase in

their ability to coprecipitate with HeV F. This suggests that there is a subtle conformational difference between a mutant G which cannot bind receptor and the WT G. Historically, there have been two competing mechanistic models of paramyxovirus glycoprotein-mediated membrane fusion (reviewed in (58)). One model suggests that F and G interact only after receptor binding takes place and presumably receptor binding triggers a conformational change in G which facilitates this F interaction. This interaction then is the fusion-promoting activity of G. Subsequently the F glycoprotein becomes fusion-activated, inserts its fusion peptide into target membranes, and facilitates the membrane fusion process. The second model suggests that interaction of the F and G envelope glycoproteins is preexisting and independent of any receptor binding event, and that it is receptor binding that triggers conformational change in G, that may or may not release F, but nevertheless triggers the fusion activity of F. The present data support this second model, in which F and G interact prior to receptor binding in that not only does WT G coprecipitate with F in the absence of receptor (HeLa-USU cells are receptor negative (7)), but also in that G glycoprotein mutants which possess significant defects in receptor binding can still coprecipitate with F to levels equivalent to or greater than that of WT G. Although we favor the interpretation that we have removed important residues in G for engaging receptor, an alternative explanation could be that some of these mutant G glycoproteins are adopting a pre-receptor bound conformation which is more favorable for F binding and less favorable for receptor binding.

The effects of several mutations in HeV G on ephrinB2 and ephrinB3 binding did translate into measurable effects on their abilities to promote cell-fusion as predicted. The overall trend observed from the present experiments was that reduced receptor

binding capacity of individual G mutants correlated qualitatively with reductions in cell fusion measurements, although we cannot exclude the possibility that the reductions in cell fusion promotion activity observed for some G mutants (D257A, D260A, K443A, G449A, and D468A) could be caused, at least in part, by those mutants' increased abilities to bind HeV F in addition to their decreased receptor binding phenotypes. Further experiments will be needed to dissect out the contributions of each of these effects on the fusion process. Taken together, the various mutations in HeV G identified here that impaired its functional activities appear specific for receptor binding and were not due to gross conformational defects, loss of an ability to interact with F, or a lower cell-surface expression phenotype.

Notably, in addition to showing that like NiV G, HeV G could engage ephrinB3 (**Fig. 2**) and use it as a functional receptor in cell fusion (data not shown), it was also noted that the same residues within HeV G which appeared critical for binding ephrinB2 were also important for binding ephrinB3. Mutation of residues D257, D260, G439, K443, G449, K465, and D468 of HeV G to alanine resulted in defects in ephrinB3 binding, similar to the binding pattern observed with ephrinB2. Together with data from Negrete et al., indicating that addition of soluble ephrinB2 can inhibit ephrinB3-dependent entry by NiV and vice versa (61), the present observations strongly suggest that each G glycoprotein binds to two different members of the ephrin ligand family via a common receptor binding domain. Further study of additional HeV as well as NiV G mutations will help better delineate all the domain(s) of G involved in receptor engagement. It will also be important to confirm these observations in the context of virus infection in future experiments. In summary, our data provide strong biochemical

and functional evidence of a conformation-dependent and/or discontinuous ephrinB2 and ephrinB3 binding domain within the henipavirus G glycoprotein and will aid our understanding of the binding and infection process of these important emerging pathogens.

Chapter 4: A stretch of hydrophobic residues between Heptad Repeats A and B of Hendra virus F is critical for fusion

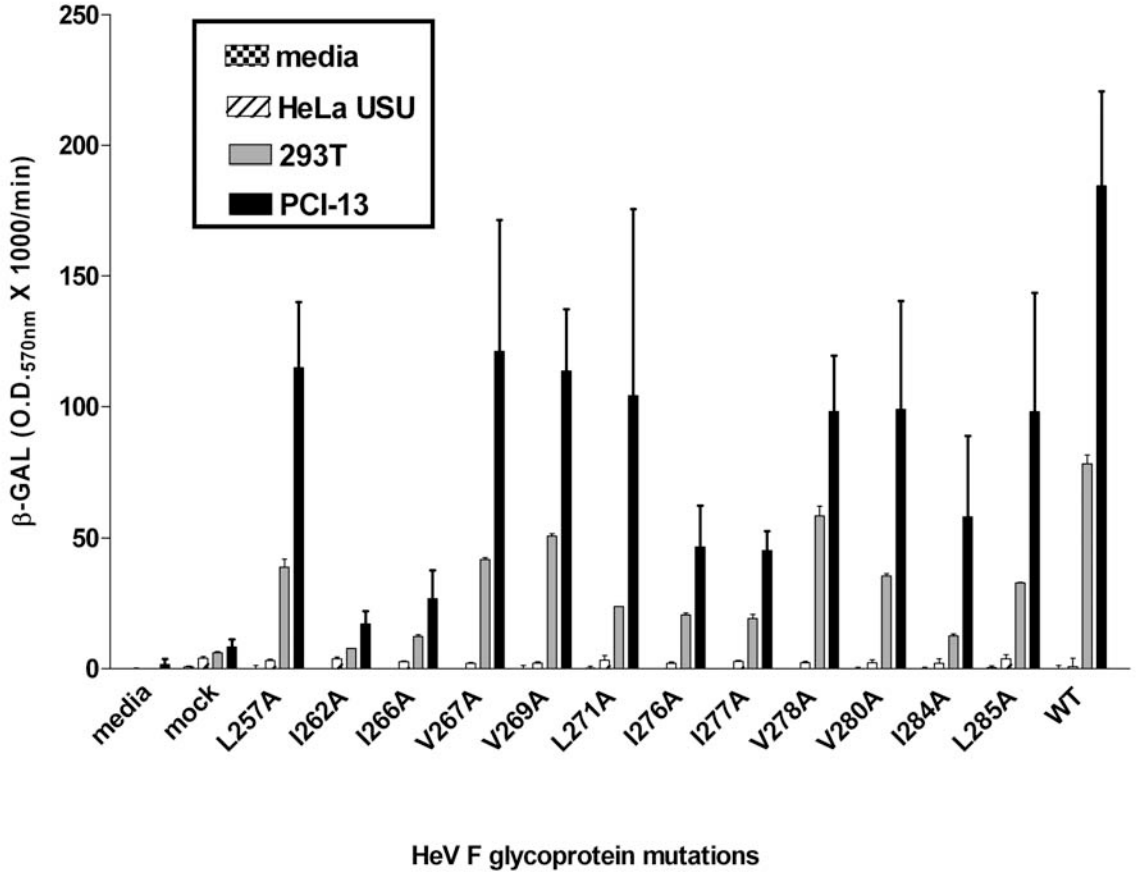
Introduction

The paramyxovirus F glycoprotein facilitates the merger of virion and host cell membranes via a pH-independent, Class I fusion during virus infection. After insertion of F's hydrophobic fusion peptide into the target cell membrane, target and virus membranes are pulled closely together through a conformational rearrangement of F into a six helix bundle structure. Six helix bundle formation occurs through interactions of two important regions of F– heptad repeats A and B (HRA and HRB). In the current study, another predicted HR domain in HeV F was examined to explore its potential role in the fusion process.

Results

Expression and fusion activity of HeV F glycoprotein single point mutants. A stretch of hydrophobic residues resembling an isoleucine zipper-like HR domain was identified in HeV F. As determined by amino acid sequence alignment (not shown), this domain, which consists of HeV F residues 257-285, is relatively well conserved among other members within the *Paramyxoviridae* family, and is also proximal to a region which in NDV F has been previously identified to be important for fusion (74). Leucine, isoleucine, and valine residues in this region were individually converted to alanine and the resulting mutants were assessed for their ability to mediate membrane fusion when coexpressed with HeV G in our quantitative cell-cell fusion assay. The results are shown

Figure 7. Fusion activity of HeV F glycoprotein mutants. The various alanine mutants or WT HeV F were coexpressed with HeV G and assayed for their fusion activity in a quantitative, reporter gene, cell fusion assay. Results averaged from 2 independent experiments, each in duplicate wells, are shown. Error bars indicate standard deviation.


media
HeLa USU
293T
PCI-13
β-GAL (O.D.570nm X 1000/min)
250
200
150
100
50
0
media
mock
L257A
I262A
I266A
V267A
V269A
L271A
I276A
I277A
V278A
V280A
I284A
L285A
WT
HeV F glycoprotein mutations

in **Fig. 7**. All the alanine mutations resulted in decreased cell fusion ability as measured by our assay, but the most substantial defects were conveyed by the mutation of isoleucine residues to alanine. All 5 isoleucine to alanine mutations each resulted in fusion activity less than 50% of WT F.

As defects in viral glycoprotein-mediated fusion activity can be brought about by defects in cell surface expression of mutant F glycoproteins, cell surface expression levels of each glycoprotein mutant were examined. HeLa-USU cells expressing the panel of mutants or WT F were surface biotinylated at 4°C for 30 minutes and lysates of the cells were prepared. Solubilized proteins were precipitated with Avidin agarose beads followed by SDS-PAGE and Western blotting with an F-specific polyclonal rabbit antiserum. As shown in **Fig. 8**, each F glycoprotein mutant was found to be expressed on the cell surface at levels similar to WT F; thus the decreased fusion phenotype possessed by these glycoproteins cannot be attributed to defects in cell surface expression.

Expression and fusion activity of HeV F glycoprotein multiple point mutants. As the most dramatic fusion defects were seen in the context of isoleucine to alanine mutations, the effects of multiple isoleucine to alanine mutations were then examined in a follow-up experiment with the expectation that at least additive negative effects on F fusion activity could be observed. Two double, two triple, and two quadruple mutants were constructed and assayed for their resulting fusion activity when coexpressed with HeV G in our quantitative cell-cell fusion assay. Not surprisingly, the presence of two or more isoleucine to alanine mutations resulted in a complete loss of fusion activity (**Fig. 9**), which further underscores the functional importance of these residues and this domain of the molecule.

Figure 8. Cell surface expression levels of HeV F glycoprotein single point mutants. The various single point mutants of F or WT F were transiently expressed in HeLa-USU cells. Surface-expressed proteins were labeled with biotin at 4°C and then precipitated with Avidin agarose beads followed by SDS-PAGE and western blotting with polyclonal antisera specific for HeV F. Total cell lysates were also probed as control for total expression levels.

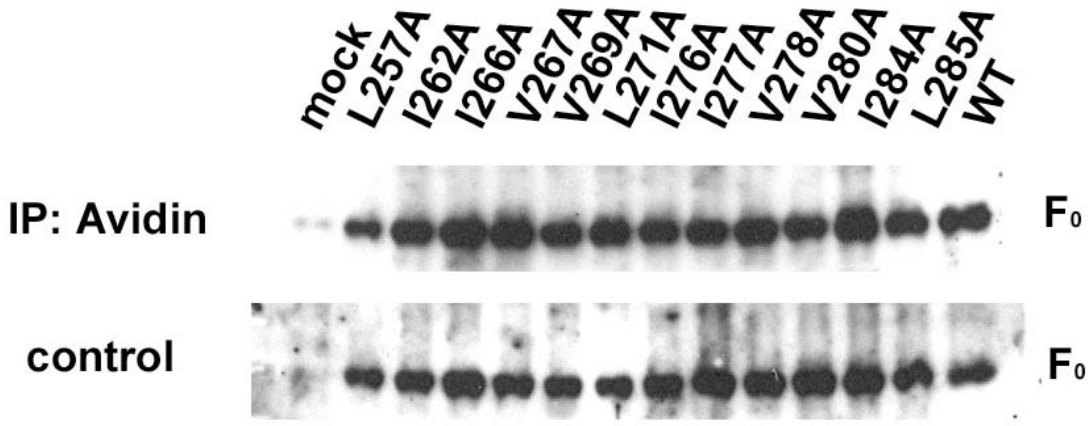
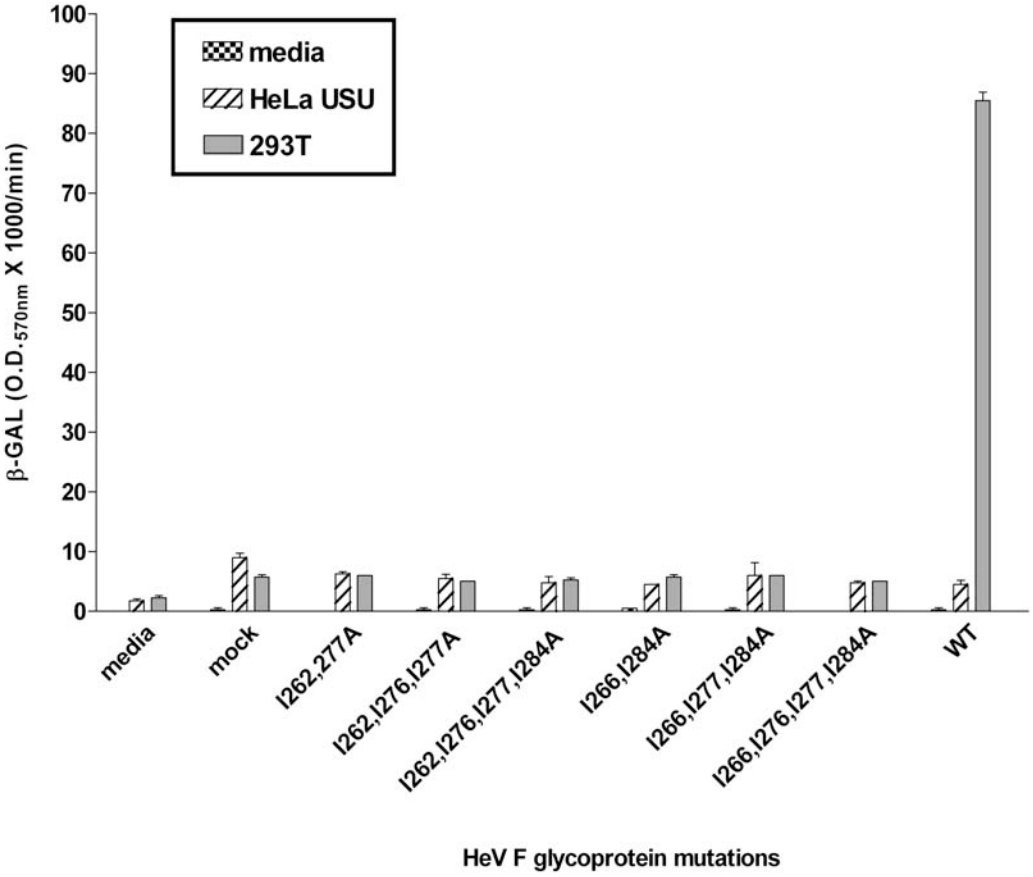

mock
L257A
I262A
I266A
V267A
V269A
L271A
I276A
I277A
V278A
V280A
I284A
L285A
WT
IP: Avidin
F0
control
F0

Figure 9. Effects of multiple point mutations on fusion activity of HeV F. Multiple point mutants of HeV F or WT F were coexpressed with HeV G and assayed for their fusion activity in a quantitative, reporter-gene cell fusion assay performed in duplicate wells. Error bars represent standard deviation.


media
HeLa USU
293T
β-GAL (O.D.570nm X 1000/min)
100
90
80
70
60
50
40
30
20
10
0
media
mock
I262,277A
I262,I276,I277A
I262,I276,I277,I284A
I266,I284A
I266,I277,I284A
I266,I276,I277,I284A
WT
HeV F glycoprotein mutations

As before, each of the multiple point mutants was also analyzed for its cell surface expression level by a surface protein biotinylation assay, and the results are shown in **Fig. 10**. Like the individual point mutants, each multiple point mutant of F was found to be expressed on the surface of cells to levels comparable to WT F, again indicating that the decreased fusion activity was a specific effect caused by the point mutations and not attributable to diminished cell-surface expression.

G-interaction ability of HeV F glycoprotein mutants. Leucine zippers are often implicated in protein-protein interactions (for an example of a viral protein that interacts with a cellular protein in this manner, see (88)). Therefore, the individual and multiple point mutants of F were next assayed for their ability to interact with and coprecipitate with their partner glycoprotein, HeV G, with the notion that the disruption of this ability may explain the fusion-defective phenotypes. The various mutants or WT F were each coexpressed with HeV G and metabolically-labeled in HeLa-USU cells. After a 1.5 hour chase to allow protein maturation and transport, cell lysates were prepared and subjected to immunoprecipitation with an F-specific polyclonal antiserum followed by SDS-PAGE and autoradiography analysis. The results are shown in **Fig. 11** and demonstrate that each mutant F glycoprotein was precipitated by the F-specific antiserum, and in addition, each mutant and WT F protein was able to coprecipitate G. These data indicate that although alanine mutations in this region of HeV F decrease in and some cases, completely abrogate fusion activity, the defect is not due to an inability to bind and interact with the partner glycoprotein, G.

Although most of the single point mutants were defective in fusion as compared to WT F they nevertheless could mediate some fusion, with certain mutants'

Figure 10. Effects of multiple point mutations on cell surface expression level of F. The various multiple point mutants or WT F were expressed in HeLa-USU cells and surface-expressed proteins were biotin-labeled at 4°C. Biotinylated proteins were precipitated with Avidin agarose beads and subjected to SDS-PAGE followed by western blotting with polyclonal F-specific antisera.

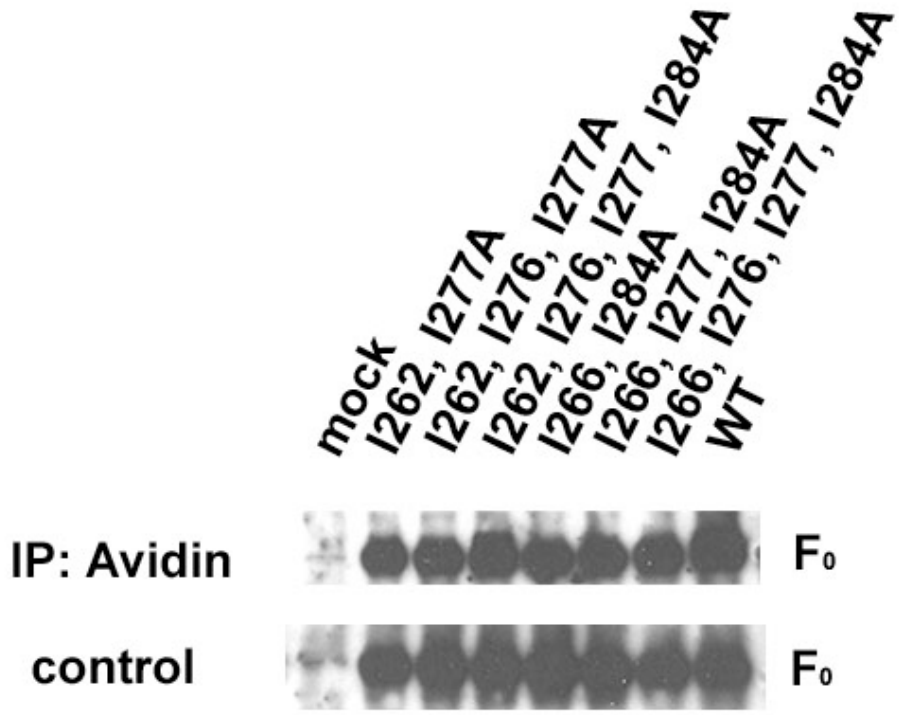
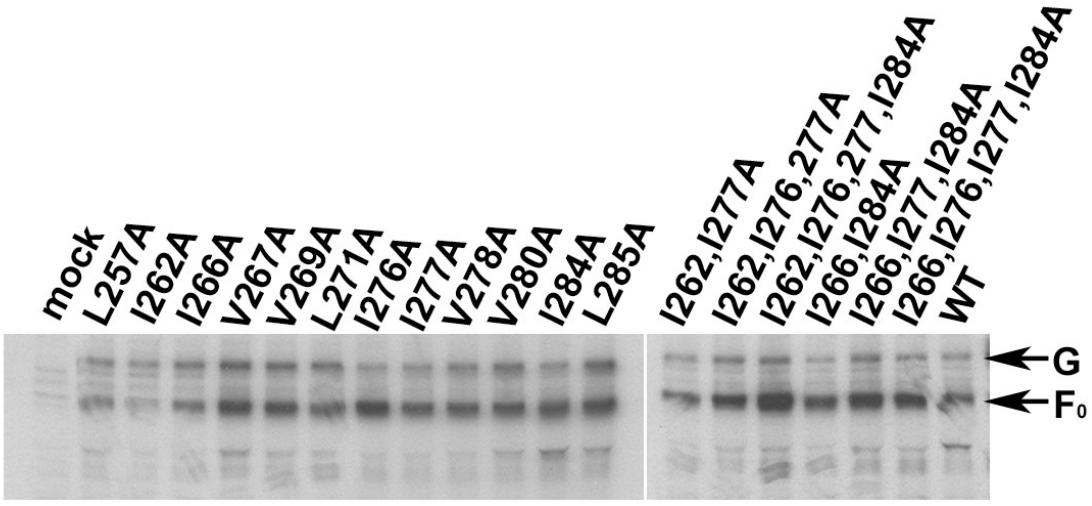

mock
I262, I277A
I262, I276, I277A
I262, I276, I277, I284A
I266, I284A
I266, I277, I284A
I266, I276, I277, I284A
WT
IP: Avidin
F0
control
F0

Figure 11. Ability of HeV F glycoprotein mutants to interact with HeV G. The various mutants or WT F were coexpressed in HeLa-USU cells, metabolically-labeled, and immunoprecipitated with F-specific polyclonal antisera in order to assess their ability to coprecipitate their partner glycoprotein, HeV G.

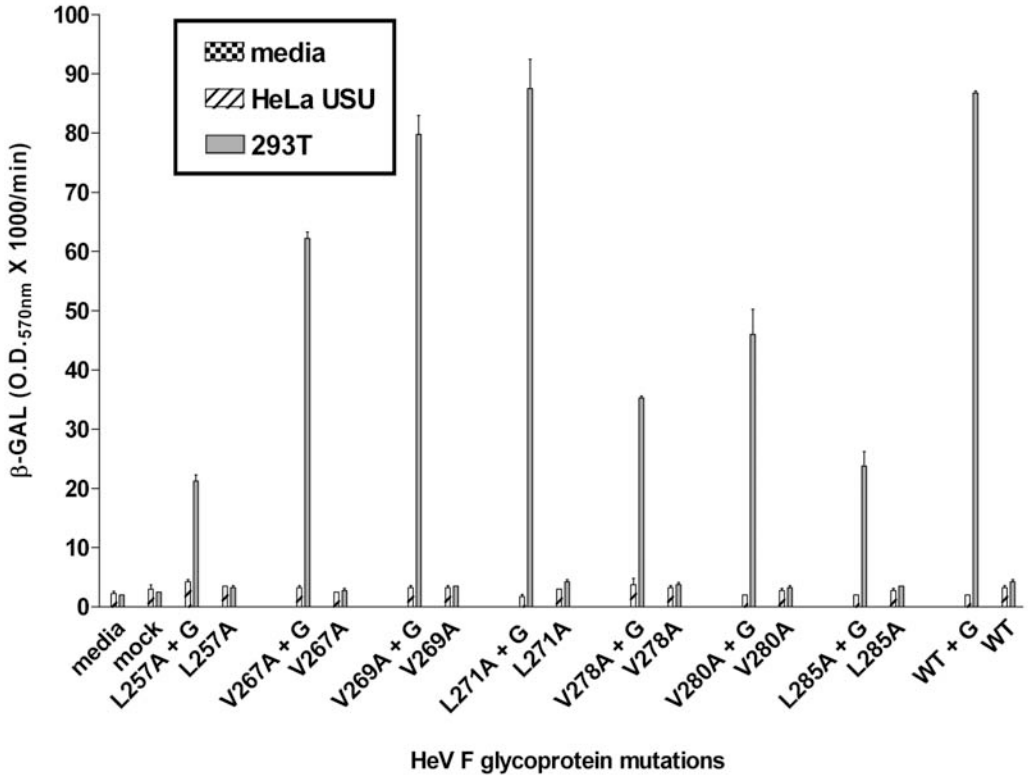

mock
L257A
I262A
I266A
V267A
V269A
L271A
I276A
I277A
V278A
V280A
I284A
L285A
I262,I277A
I262,I276,277A
I262,I276,277,I284A
I266,I284A
I266,I277,I284A
I266,I276,I277,I284A
WT
G
F0

fusion activity reduced by 30-50%. Therefore, an interesting question was whether this level of F-mediated fusion was the result of G independence by disruption of this apparently important domain of the F glycoprotein. A previous report demonstrated that a single point mutation, L289A, of the NDV F glycoprotein was found to confer upon F the ability to mediate cell-cell fusion in the absence of the partner HN glycoprotein (74). To explore the potential of such a change here, a subset of the F mutant panel was next assayed for fusion in the presence and absence of G. Each single leucine or valine to alanine substitution of HeV F was introduced into HeLa-USU cells as described in the Methods, along with either HeV G encoding or empty vector. The resulting HeV glycoprotein-expressing effector populations were assessed for their cell fusion activity and the results are shown in **Fig. 12**. In contrast to NDV F, none of the HeV F mutants was able to mediate fusion in the absence of the partner glycoprotein, HeV G.

Discussion

Heptad repeats in general and leucine zippers in particular are critical to the fusion activity of several paramyxovirus F glycoproteins and to that of other Class I fusion proteins, such as HIV Env, for example (44). In the current study, a potential isoleucine-zipper domain of the HeV F glycoprotein was identified and through amino acid sequence alignment with other paramyxovirus F proteins, found to be very well conserved across viral species. This previously unexplored domain of HeV F was investigated here through a site-directed mutagenesis strategy targeting several hydrophobic residues in a region between amino acid residues 257-285, and by assessing its contribution to fusion. Not surprisingly, upon mutation of each single leucine,

Figure 12. Alanine mutants of HeV F cannot mediate fusion in the absence of HeV G. Plasmids encoding the various leucine or valine to alanine mutants of HeV F were cotransfected into HeLa-USU effector cells along with either HeV G or empty vector plasmid and tested for their ability to mediate fusion in our cell-cell fusion assay. Error bars illustrate standard deviation.


media
HeLa USU
293T
β-GAL (O.D.570nm X 1000/min)
100
90
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50
40
30
20
10
0
media
mock
L257A + G
L257A
V267A + G
V267A
V269A + G
V269A
L271A + G
L271A
V278A + G
V278A
V280A + G
V280A
L285A + G
L285A
WT + G
WT
HeV F glycoprotein mutations

isoleucine, and valine residue in this region to alanine, there was a notable reduction in the fusion activity of F. Specifically, the most severe defects were observed upon mutation of any isoleucine residue to alanine. In addition, conversion of multiple isoleucine residues to alanine resulted in a complete ablation of fusion activity. The finding that residues within this region of HeV F are critical for fusion is consistent with the work of Sergel et al., who demonstrated that mutation of residues L268 and L289 of the NDV F glycoprotein had a similar effect (74), although there were also important distinctions between NDV F and HeV F, data which are discussed below.

Notably, the observed defects in fusion activity of the single point mutants and multiple point mutants were not attributable to defects in cell-surface expression. This is an important observation, as mutagenesis of some other well-conserved regions of paramyxovirus F glycoproteins in other studies has been shown to result in defects in glycoprotein trafficking, folding, and subsequent cell-surface expression (32, 67). In addition, the observed defects in fusion were not due to any failure of the F mutants to interact with their partner glycoprotein, HeV G, as all mutants of F retained this ability and could coprecipitate HeV G. Unfortunately, these results also indicated that this region of the F glycoprotein is unlikely to be a G-interaction domain– which still remains to be identified.

A particularly interesting finding of the current study is that none of the alanine mutations made in HeV F resulted in attachment glycoprotein independence, even though a similar mutation, L289A, in NDV F was previously found by Sergel and colleagues to confer upon NDV F the ability to promote membrane fusion in the absence of NDV HN (74). The finding that none of the HeV F mutants were fusogenic in the absence of G

was surprising, as amino acid sequence alignment of HeV F with the other paramyxovirus F glycoproteins suggests that there is much homology in this domain. The finding of Sergel and colleagues is suggestive that either a) the region of NDV F around residue L289 could be an NDV HN interaction domain or b) the region of NDV F around residue L289 could be a part of F which is involved in maintenance of the metastable state, with mutation of this residue destabilizing the F glycoprotein in a manner that allows spontaneous triggering of fusion activity. Either way, the findings of the current study suggest that there are significant differences that exist between NDV F and HeV F, and that this domain may operate differently between the two F glycoproteins.

Recently, the solved structures of two paramyxovirus F glycoproteins have been published– one of which is thought to resemble the post-fusion conformation and the other to resemble the prefusion conformation of F (94, 95). Based on these solved structures and amino acid alignments of HeV F with other paramyxovirus F glycoproteins, the residues investigated in the current study are predicted to lie within the DIII region of F's "neck" domain. Interestingly, based on the solved structures, it is modeled that this DIII region undergoes a dramatic conformational alteration during the transition from the prefusion to the post-fusion form of F. In fact, it is believed that these conformational changes actually require DIII to rotate and collapse inward (44). This suggests that the DIII region plays a critical role in F protein structure and folding, and could explain why alanine mutations made within this region have such drastic effects on cell fusion, while having little effect on the other characteristics of F. Indeed, the hPIV-3 F structure shows that paramyxovirus F residues identified as being important for fusion

in previous studies actually lie along the interface of the globular head DI region with the DIII neck region (94).

Currently, our laboratory is working to develop murine and human monoclonal antibodies to HeV F. Once generated, such antibodies will be exceedingly useful for comparison of the conformation of F glycoprotein mutants versus that of WT F. Such experiments should help define in greater detail exactly how the point mutations between residues 257 and 285 are affecting or inhibiting the triggered refolding of F. Despite our current lack of antibodies for analyzing F, however, the current study clearly demonstrates that residues 257-285 are critical to the fusogenic activity of the HeV F glycoprotein. Additionally, these data support the hypothesis that the DIII region of F is important in refolding from the prefusion to the post-fusion F. Further examination of these F mutants in the future with additional tools should provide useful information.

Chapter 5: Residues in the stalk domain of Hendra virus G glycoprotein modulate conformational changes associated with receptor binding

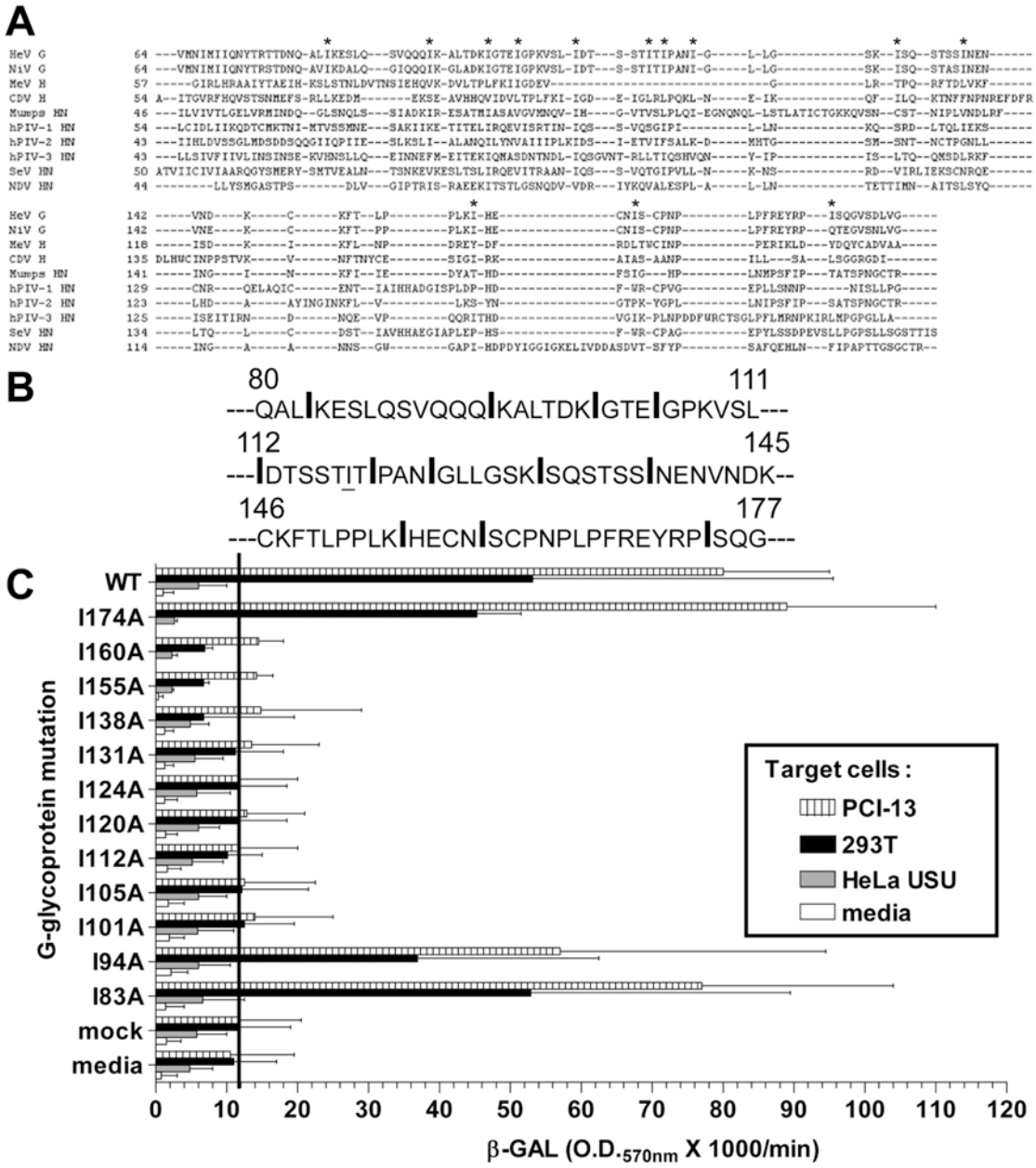
Introduction

The stalk region of the paramyxovirus attachment protein has been implicated in such various functions as F interaction/specificity and oligomerization of H. In the current study, a conserved domain within the stalk region of HeV G was identified and through site-directed mutagenesis, examined for its contribution to the various structural and functional characteristics of G, including fusion-promotion activity. Interestingly, mutations in this domain were found to trigger spontaneous conformational changes normally associated only with receptor-bound G.

Results

Expression and fusion-promotion ability of HeV G glycoprotein stalk mutants. An isoleucine-rich stretch of residues in the HeV G glycoprotein stalk domain was identified that is also 100% conserved in NiV G and well conserved, with either isoleucine, leucine, or valine residues at analogous positions, amongst several other paramyxoviruses, including the HN glycoprotein of NDV, Sendai virus (SeV), human parainfluenza virus-1 (hPIV-1) and hPIV-3, and the H glycoprotein of MeV and Canine Distemper Virus. An amino acid sequence alignment of the stalk region of the n-terminal part of HeV G with that of other paramyxovirus attachment proteins is shown in **Fig. 13A**. Earlier work suggested that these residues in NDV HN have been proposed to form two heptad repeat like structures similar to those in the F glycoprotein (76),

Figure 13: Fusion promotion activity of HeV G glycoprotein stalk mutants. In A) is a partial sequence alignment of the HeV G stalk domain with that of other Paramyxovirus attachment proteins. Asterisks (*) indicate the motif of conserved isoleucine, leucine, and valine residues. In B) is depicted that particular motif in the stalk of HeV G consisting solely of isoleucines. Large, bolded residues are those which were mutated to alanine and subsequently assessed for fusion promotion activity in the current study. The I118 residue which we were unable to mutate is shown in plain unbolded, text and underlined. In C) the various HeV G alanine mutants or WT HeV G were coexpressed with HeV F and assayed for their ability to promote cell-cell fusion when mixed with receptor-positive 293T or PCI-13 cells in a quantitative, vaccinia virus-based fusion assay as described in Materials and Methods. HeLa-USU cells, which are receptor-negative for Henipaviruses, served as a negative control. The means of two independent experiments are shown. Error bars illustrate range.


A
HeV G
NiV G
MeV H
CDV H
Mumps HN
hPIV-1 HN
hPIV-2 HN
hPIV-3 HN
SeV HN
NDV HN
B
80
111
---QAL|KESLQSVQQQ|KALTDK|GTE|GPKVSL---
112
145
---|DTSSTIT|PAN|GLLGSK|SQSTSS|NENVNDK--
146
177
---CKFTLPPLK|HECN|SCPNPLPFREYRP|SQG---
C
WT
I174A
I160A
I155A
I138A
I131A
I124A
I120A
I112A
I105A
I101A
I94A
I83A
mock
media
G-glycoprotein mutation
Target cells :
PCI-13
293T
HeLa USU
media
0
10
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40
50
60
70
80
90
100
110
120
β-GAL (O.D.570nm X 1000/min)

and that this stalk region was important for interaction between the attachment glycoprotein and F and in determining F-virus species fusion specificity (26, 35, 79, 81). Molecular modeling of HeV G shows this region of the protein stalk as an alpha-helical stretch and in some configurations the domain possesses a kink. To explore whether this domain in HeV G possessed similar functional importance, 12 of 13 isoleucine residues from amino acid position 83 to 174 in HeV G were individually mutated (targeted residues are depicted in **Fig. 13B**) and the mutant G glycoproteins were assessed for their fusion-promotion activity.

Each of the HeV G mutation containing constructs or WT G was coexpressed with HeV F in an effector cell population and then tested for its ability to promote membrane fusion with a receptor-bearing partner cell population, and the results are shown in **Fig. 13C**. This analysis revealed that 9 of 12 G glycoprotein mutants had completely lost the ability to promote membrane fusion. Notably, all 9 of these 12 isoleucine residues were better conserved overall among the majority of the attachment glycoproteins analyzed by sequence alignment than the 3 isoleucine residues (I83, I94 and I174) which had no effect on the glycoprotein's fusion-promotion activity when altered and which were located at the terminal ends of the domain of interest. These single amino acid substitutions in G were profoundly defective in supporting fusion but the nature of this defect was unknown. Relative temperature has been shown to effect viral glycoprotein-mediated cell-cell fusion; however the block in fusion caused by these mutations could not be overcome by increasing the temperature at which the fusion assay was conducted from 37°C to 42° C (data not shown).

Although it seemed unlikely that these individual alanine substitutions would globally disrupt an important overall structure possessed by this stalk domain, the next question to ask was whether the observed fusion defect could be due to gross misfolding of the mutant glycoproteins and thus significantly reducing expression levels at the cell surface. Nevertheless, subtle changes in cell-surface expression levels of viral membrane glycoproteins that are involved in membrane fusion can have measurable effects on the fusion process. In order to assess the surface expression of these mutants, the surfaces of cells transiently expressing either each mutant or WT G were biotinylated at 4°C. After washing to remove excess biotin, cells were lysed and surface-expressed proteins were then precipitated using avidin beads. Under these conditions, only two of the mutants (I155A and I160A) exhibited decreased levels of cell surface expression in comparison to wild-type HeV G although they were readily detected in the cell lysates (**Fig. 14**). Thus, the defect in fusion-promotion ability conveyed by the majority of the mutants (7 of 9) could not be attributed to gross misfolding of the protein and resultant defects in cell-surface expression.

Receptor binding and F-engagement by G glycoprotein stalk mutants. Loss or defects in receptor binding ability will have a significant effect on the G glycoprotein's fusion-promotion activity. Therefore, each of the G glycoprotein mutants was next tested for the ability to bind the henipavirus receptors ephrinB2 and ephrinB3, using a coprecipitation-based assay (7). Using this assay, each of the mutants, including I155A and I160A which were poorly surface expressed, were coprecipitated with soluble, epitope-tagged human ephrinB2 and ephrinB3 to levels equivalent to WT HeV G. These results shown in **Fig. 15** demonstrate that the defect in

Figure 14: Cell surface expression of HeV G glycoprotein stalk mutants. Proteins on the surfaces of HeLa-USU cells transiently expressing HeV G alanine mutants or WT HeV G were biotin-labeled at 4°C. Lysates were prepared and biotin-labeled proteins were immunoprecipitated with avidin-agarose beads, subjected to SDS-PAGE, and immunoblotted with G-specific antisera as described in Materials and Methods. Total cell lysates were also probed with polyclonal G-specific antisera for comparison (control).

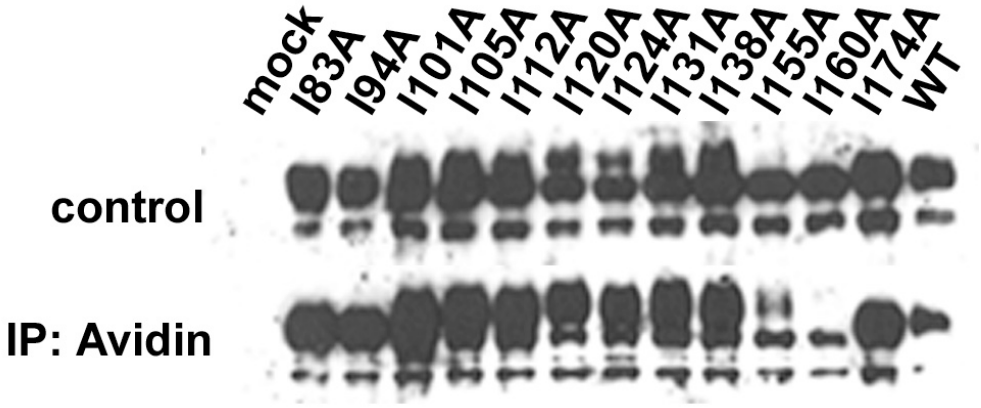
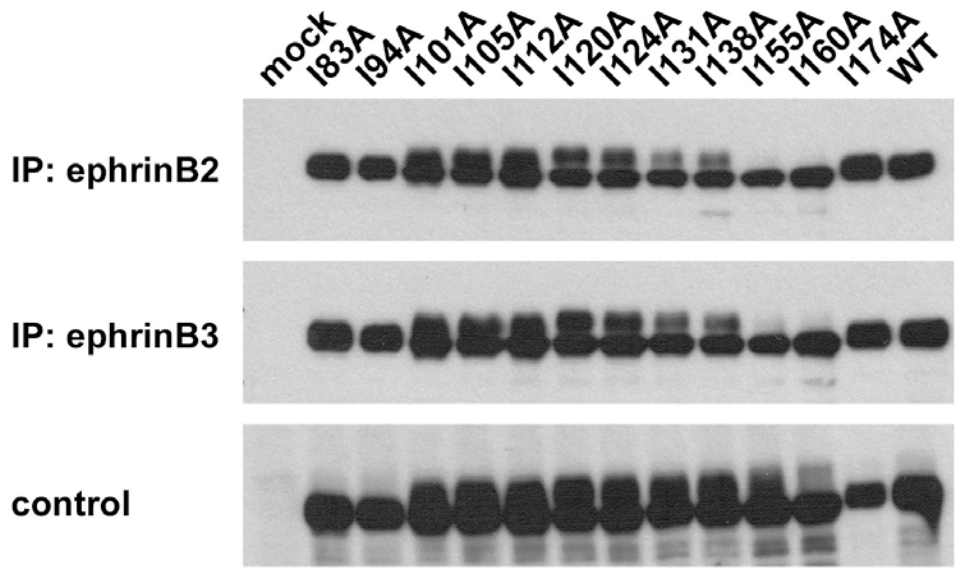

mock
I83A
I94A
I101A
I105A
I112A
I120A
I124A
I131A
I138A
I155A
I160A
I174A
WT
control
IP: Avidin

Figure 15: Receptor binding by HeV G glycoprotein stalk mutants. The various alanine mutants or WT HeV G were transiently expressed in HeLa-USU cells and subjected to coprecipitation with s-tagged human ephrinB2 followed by S-agarose beads or FC-tagged human ephrinB3 followed by Protein G beads. Each lysate was also directly precipitated with polyclonal G-specific antisera followed by Protein G beads for comparison (control). Precipitated proteins were analyzed by SDS-PAGE followed by western blotting with polyclonal G-specific antisera.


mock
I83A
I94A
I101A
I105A
I112A
I120A
I124A
I131A
I138A
I155A
I160A
I174A
WT
IP: ephrinB2
IP: ephrinB3
control

fusion-promotion activity caused by these mutations is not attributable to lack of receptor binding competence by the mutants.

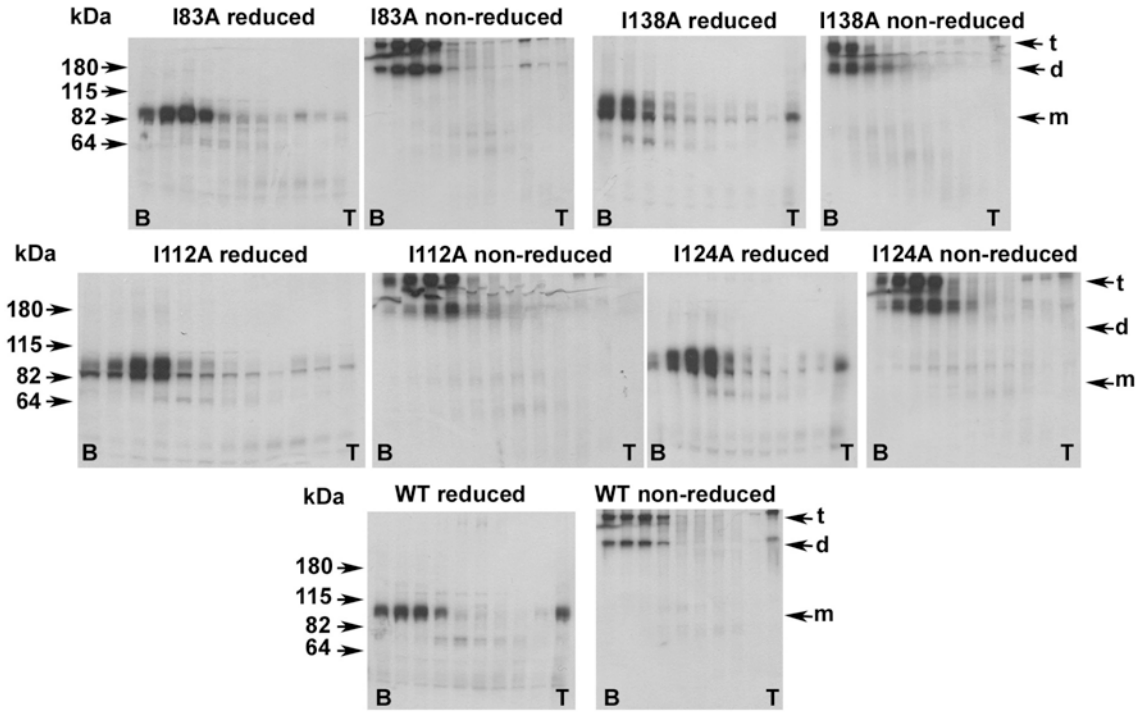
Previous work carried out with attachment glycoproteins of other paramyxoviruses containing mutations in the stalk domain has implicated this region as an important determinant of F-engagement and F species-specificity. Therefore, we next assessed whether these HeV G stalk domain mutants which possessed impaired fusion-promotion activity could be related to a decreased ability to interact with their partner glycoprotein, HeV F. Using our previously developed coprecipitation-based assay for F/G engagement, we found no correlation between defects in fusion-promotion ability to any decreased ability to interact with the F glycoprotein (results not shown).

Oligomerization of HeV G glycoprotein stalk mutants. Previous work has demonstrated that paramyxovirus attachment glycoproteins appear to be disulfide-linked dimers and that the native configuration appears to be a dimer of dimers or tetrameric (10). In addition, the stalk domain of MeV H is a critical determinant of H oligomerization (68), thus the possibility that the HeV G stalk mutants generated here could be defective in oligomerization and as a result be defective in their fusion-promotion activity needed to be examined. Therefore, several of the HeV G glycoprotein stalk mutants were next analyzed for their ability to form the dimeric and tetrameric species similar to that of WT HeV G. Several of the completely fusion-defective mutants and one fusion-competent stalk mutant as well as WT G were each transiently expressed, metabolically-labeled and cell-lysates prepared and subjected to sucrose density ultracentrifugation, fractionation, and immunoprecipitation with G-specific antisera. The immunoprecipitated G glycoproteins in each fraction of the gradient were analyzed by

SDS-PAGE under reducing and non-reducing conditions, and these results are shown in **Fig. 16.** This analysis revealed that each HeV G mutant examined was observed to form oligomers with an identical pattern exhibited by the WT HeV G glycoprotein, and both tetrameric and dimeric species were observed under non-reducing conditions which were resolved to monomeric forms of G under reducing conditions (**Fig. 16**), essentially identical to prior observations (10). Thus, the conclusion from this analysis is that the observed fusion-promotion defect caused by the isoleucine to alanine mutations in the stalk domain can not be attributed to a failure of these mutant G glycoproteins to form native oligomeric complexes.

Glycosylation of HeV G glycoprotein mutants. It was noted that the 9 fusion-promotion defective stalk mutants of HeV G generated here all exhibited a slower migrating species upon SDS-PAGE analysis in addition to a species that co-migrated with WT HeV G. The difference in size between this slower migrating band and the WT species was approximately 4-5 kDa and potentially attributable to an additional post-translational N-glycosylation of the glycoprotein. Therefore, we next sought to determine whether this higher molecular weight species could be due to an extraneous glycosylation in HeV G that was somehow brought about by the introduced mutations within the stalk domain. In order to test this possibility, two fusion-defective mutants as well as the WT G glycoprotein were subjected to PNGase treatment (to remove N-linked glycans) for 0, 10, and 60 minutes, and the results of the 0 and 10 minute incubation periods are shown in **Fig. 17A.** After either 10 or 60 minutes incubation with PNGase at 37ºC, there was an apparent shift in molecular weight for the two mutants and the WT G, indicating the successful removal of N-glycans from G. Importantly, after N-glycan

Figure 16: Oligomerization ability of HeV G glycoprotein stalk mutants. Various alanine mutants or WT HeV G were transiently expressed in HeLa-USU cells, metabolically labeled, and chased as described in Materials and Methods. Each lysate was layered onto a continuous 5-20% sucrose gradient and centrifuged at 40,000 rpm for 20 hr at 4ºC. Then each gradient was fractionated and immunoprecipitated with polyclonal G-specific antisera and analyzed by SDS-PAGE under reducing and non-reducing conditions followed by autoradiography.


kDa
I83A reduced
I83A non-reduced
I138A reduced
I138A non-reduced
180
115
82
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I112A non-reduced
I124A reduced
I124A non-reduced
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kDa
WT reduced
WT non-reduced
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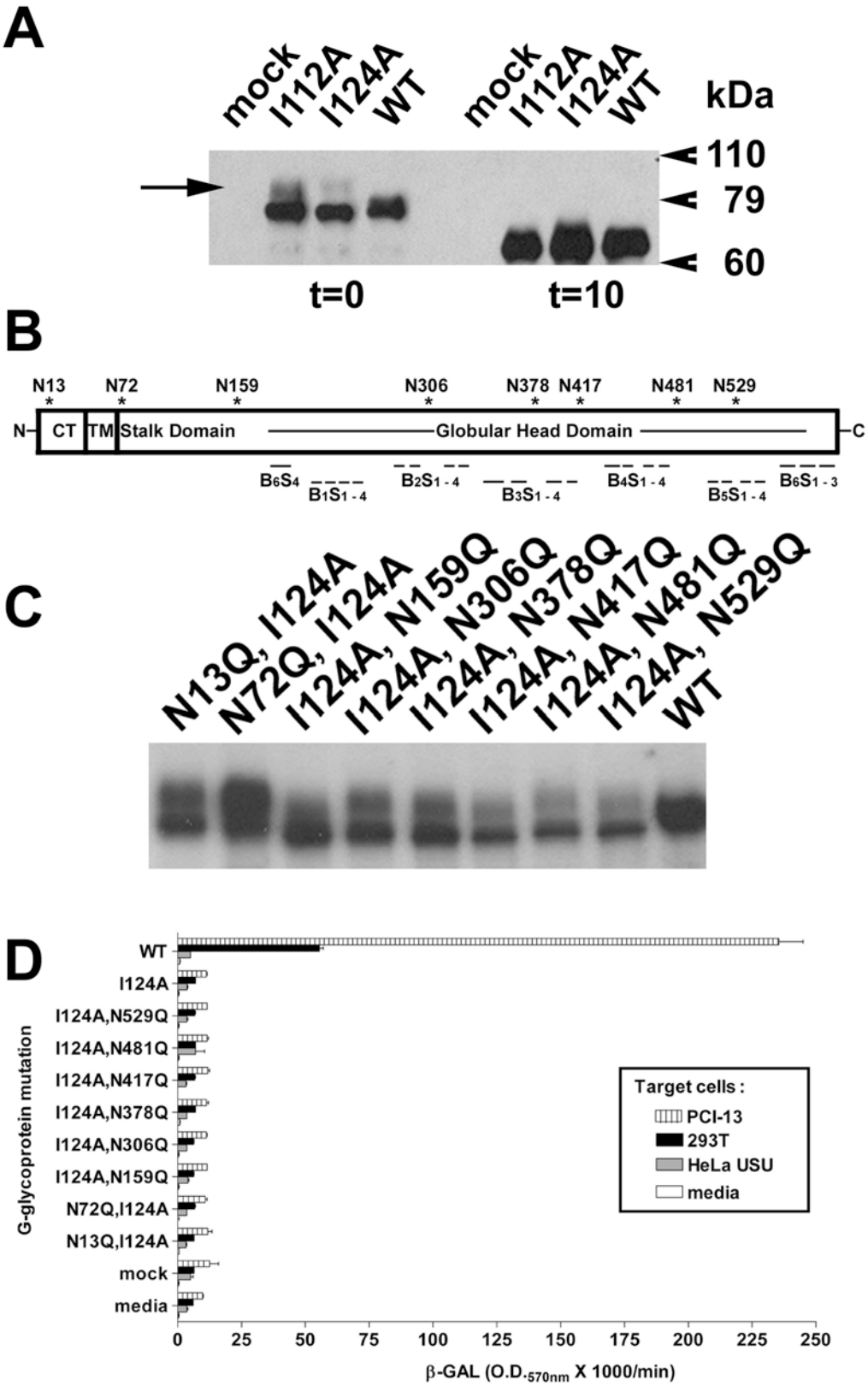
T

Figure 17: Super-glycosylation of HeV G glycoprotein stalk mutants. The apparent higher molecular weight species of fusion-defective G stalk mutants was explored. In A) G alanine mutants or WT HeV G were expressed in HeLa-USU cells and lysates prepared as described in the Methods. G-containing lysates were immunoprecipitated with polyclonal G-specific antisera followed by Protein-G beads**.** Precipitated proteins were treated with PNGase F, an enzyme that removes N-linked glycans, at 37ºC and the reactions were analyzed by SDS-PAGE followed by western blotting with polyclonal G-specific antisera. In B) is a schematic of the HeV G glycoprotein illustrating the location of 8 potential N-linked glycosylation sites in reference to the stalk domain of G. In C) each potential N-linked glycosylation site was individually mutated in the I124A HeV G mutant and the resulting double mutants were tested for expression. HeLa-USU cells were transiently transfected, metabolically labeled, and chased as described in the Methods. Resulting cell lysates were immunoprecipitated with several different monoclonal and polyclonal antibodies, and the results obtained with m101 are shown for example. In D) the effect of individual glycosylation site deletions on fusion-promotion ability of G was assessed in a quantitative, vaccinia-virus based cell fusion assay as described in the Methods. The reactions were conducted in duplicate wells using receptor-positive 293T cells and receptor-negative HeLa-USU cells. Error bars illustrate range.


A
mock
I112A
I124A
WT
mock
I112A
I124A
WT
kDa
110
79
60
t=0
t=10
B
N13
N72
N159
N306
N378
N417
N481
N529
N
CT
TM
Stalk Domain
Globular Head Domain
C
B6S4
B1S1 - 4
B2S1 - 4
B3S1 - 4
B4S1 - 4
B5S1 - 4
B6S1 - 3
C
N13Q, I124A
N72Q, I124A
I124A, N159Q
I124A, N306Q
I124A, N378Q
I124A, N417Q
I124A, N481Q
I124A, N529Q
WT
D
G-glycoprotein mutation
WT
I124A
I124A,N529Q
I124A,N481Q
I124A,N417Q
I124A,N378Q
I124A,N306Q
I124A,N159Q
N72Q,I124A
N13Q,I124A
mock
media
Target cells :
PCI-13
293T
HeLa USU
media
0
25
50
75
100
125
150
175
200
225
250
β-GAL (O.D.570nm X 1000/min)

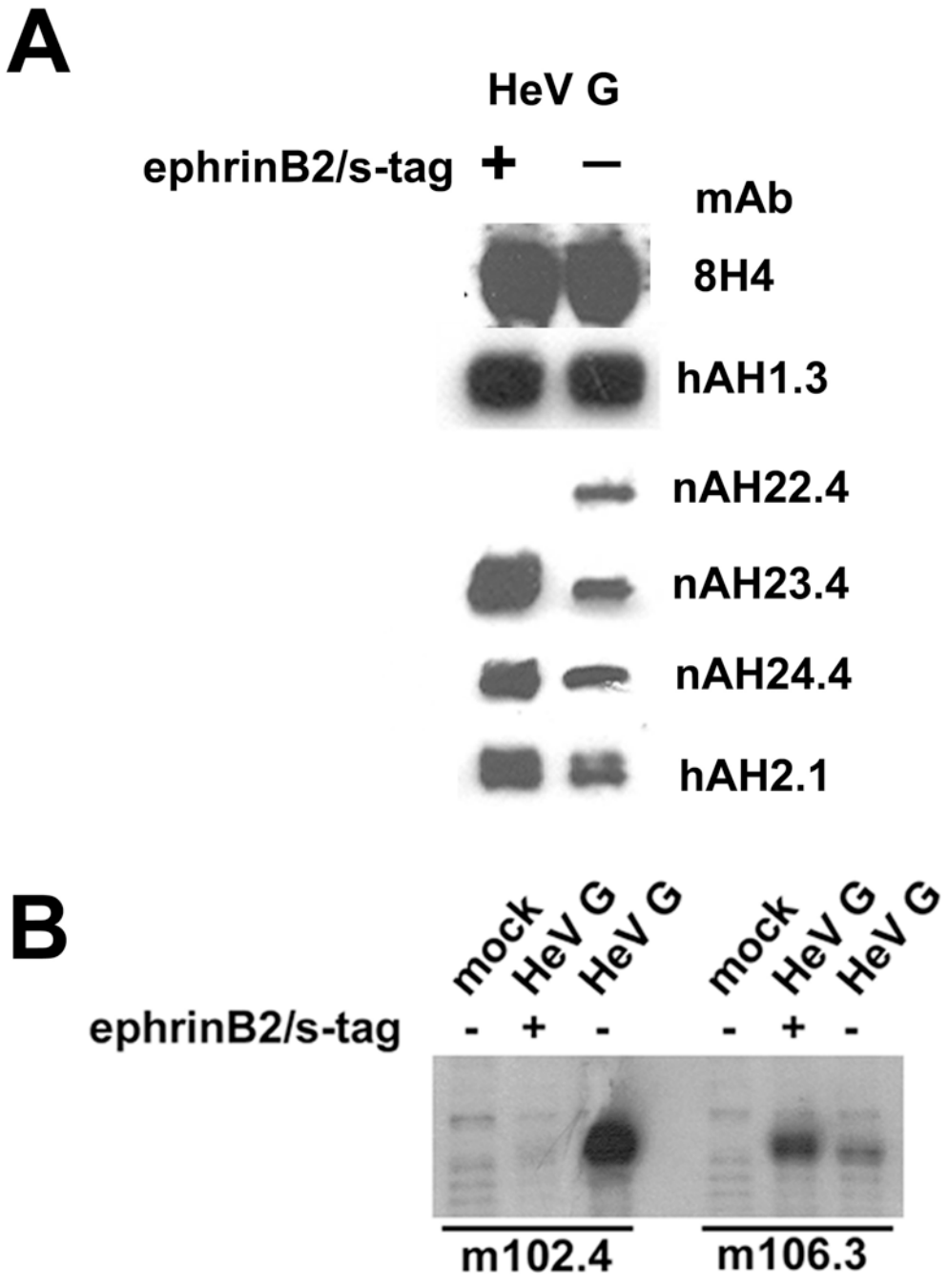
removal, there was no longer any difference in the apparent molecular weight or migratory properties between the mutants or WT G, indicating that the observed difference in migratory properties at t=0 is indeed due to glycosylation.

Interestingly, it has been reported that addition of N-glycans to paramyxovirus F or G glycoproteins can result in decreased fusion (1, 57, 85). Therefore, it was possible that this extraneous glycosylation associated with the fusion-defective stalk mutants could be covering an important domain of G involved in fusion. The HeV G glycoprotein has eight potential N-linked glycosylation sites, although exactly which sites are modified is unknown. One site is located in the cytoplasmic region of G, and another one is located in the predicted extracellular region but proximal to the predicted transmembrane anchor and likely not modified. The other six sites, however, are located at various positions along the predicted extracellular portion of G. The location of these potential sites in relation to the rest of the protein is indicated by asterisks in **Fig. 17B**. We speculated that if the presence of an extra N-glycan was occluding an important domain of G that was required for its fusion-promoting activity, then its removal by mutation should prevent this interference. Thus, to elucidate whether an extraneous glycosylation at any of these potential N-linked glycosylation sites was causing the observed block in fusion, the asparagine at each site was individually mutated to glutamine in the context of the I124A mutation, and the resulting double mutants were tested for expression. Surprisingly, not one of the glycosylation site deletions resulted in a WT electrophoretic mobility pattern, although for some of the mutants, the upper, apparently more glycosylated band in the doublet decreased somewhat in intensity (**Fig. 17C**). These 8 additional HeV G double mutants were next tested for their fusion-

promotion activity when coexpressed with F; the results of this analysis are shown in **Fig. 17D**. Although all of the double mutants were expressed at levels roughly equivalent to WT HeV G, none of these additional double mutants were rescued in their ability to promote fusion. These results demonstrated that although it is was not apparent which potential N-glycosylation site was being used for any extraneous N-glycosylation, this post-translational modification itself was not likely to be obscuring an important domain of G resulting in the observed defect in fusion-promotion activity.

Characterization of HeV G glycoprotein mutants by mAb reactivity. As an alternative method to evaluate the effects of the stalk domain mutations on the structural and functional properties of the HeV G glycoprotein, a panel of G-specific mAbs including those which compete for receptor (ephrinB2) and others that do not (87, 98) was utilized. The mAb binding reactivities of WT HeV G glycoprotein in the presence and absence of ephrinB2 were first assessed using a coprecipitation assay and these results are shown in **Fig. 18**. Three types of relative reactivity were observed: mAbs that could bind and precipitate G to equivalent levels in the presence or absence of receptor (hAH1.3 and 8H4), those that bound less because they compete for receptor (nAH22.4 and m102.4), and interestingly, mAbs that bound and precipitated G better following ephrinB2 binding (nAH23.4, nAH24.4, hAH2.1, and m106.3) – indicative of a conformational change in the G glycoprotein following receptor engagement. Interestingly, m106.3 is an affinity-matured derivative of the human Fab, m106, and so m106 was also tested in this assay and found to exhibit an identical binding

<u>Figure 18:</u> MAbs recognize receptor-induced conformational changes in HeV G. Available mAbs were assessed for their ability to recognize and immunoprecipitate HeV G glycoprotein following a 1 hr pre-incubation of G protein with either s-tagged human ephrinB2 or an equal amount of PBS at 37°C. "+" indicates incubation with ephrinB2, while "–" indicates incubation with PBS as control. In A) HeV G was precipitated with various mouse mAbs and analyzed by SDS-PAGE followed by western blotting with polyclonal G-specific antisera. In B) metabolically-labeled HeV G was precipitated with human mAbs and analyzed by SDS-PAGE followed by autoradiography.


A
HeV G
ephrinB2/s-tag + —
mAb
8H4
hAH1.3
nAH22.4
nAH23.4
nAH24.4
hAH2.1
B
mock
HeV G
HeV G
mock
HeV G
HeV G
ephrinB2/s-tag
- + - - + -
m102.4
m106.3

pattern to receptor-bound G as the affinity-matured antibody (data not shown). Therefore, m106 and m106.3 were both used in the remainder of this study.

Using this assay, cell lysates containing the various G glycoprotein mutants or WT G were immunoprecipitated with each mAb in the absence of receptor and the levels of precipitated G glycoprotein were then quantitated by densitometry. A representative result obtained using 3 conformation-dependent mAbs is shown in **Fig. 19,** and a summary of all the mAb reactivity data obtained with these HeV G glycoprotein mutants is presented in **Table 7**. All of the HeV G mutants were recognized by all conformation-dependent mAbs tested, which together with the cell-surface expression data shown earlier is further evidence that the G glycoprotein mutants are not significantly misfolded. However, an interesting pattern in mAb reactivity emerged, in that antibodies that were capable of detecting conformational changes in G following receptor binding by exhibiting increased binding reactivity also exhibited increased binding reactivity to the fusion-promotion defective stalk domain mutants of HeV G *yet in the absence of bound ephrinB2 receptor* (ie. m106, hAH2.1, nAH23.4, and nAH24.4). These data suggested that the stalk domain mutations cause conformational alterations in G that are normally associated with ephrinB2 receptor binding.

Although the possibility that these differential binding reactivities were due to actual epitope alteration seemed unlikely, the fact that nAH23.4 and nAH24.4 are western positive mAbs (data not shown) prompted us to further explore whether it was the mAbs' epitopes or G's conformation that had been altered. In order to do so, a simple boiling experiment was conducted. One would expect that if indeed our mutations had altered a shared epitope of these antibodies and the epitope were completely linear, then

<u>Figure 19:</u> Human mAb reactivities to HeV G glycoprotein stalk mutants. In A) each alanine mutant of HeV G was transiently expressed in HeLa-USU cells, metabolically labeled, and chased as described in the Methods. Lysates were divided equally into 4 parts, which were immunoprecipitated with human mAbs 101, 102.4, or 106, or rabbit polyclonal G-specific antisera as control for expression. Precipitated proteins were analyzed by SDS-PAGE followed by autoradiography. In B) resulting autorads were quantitated via spot densitometry and the results expressed as "% WT reactivity."

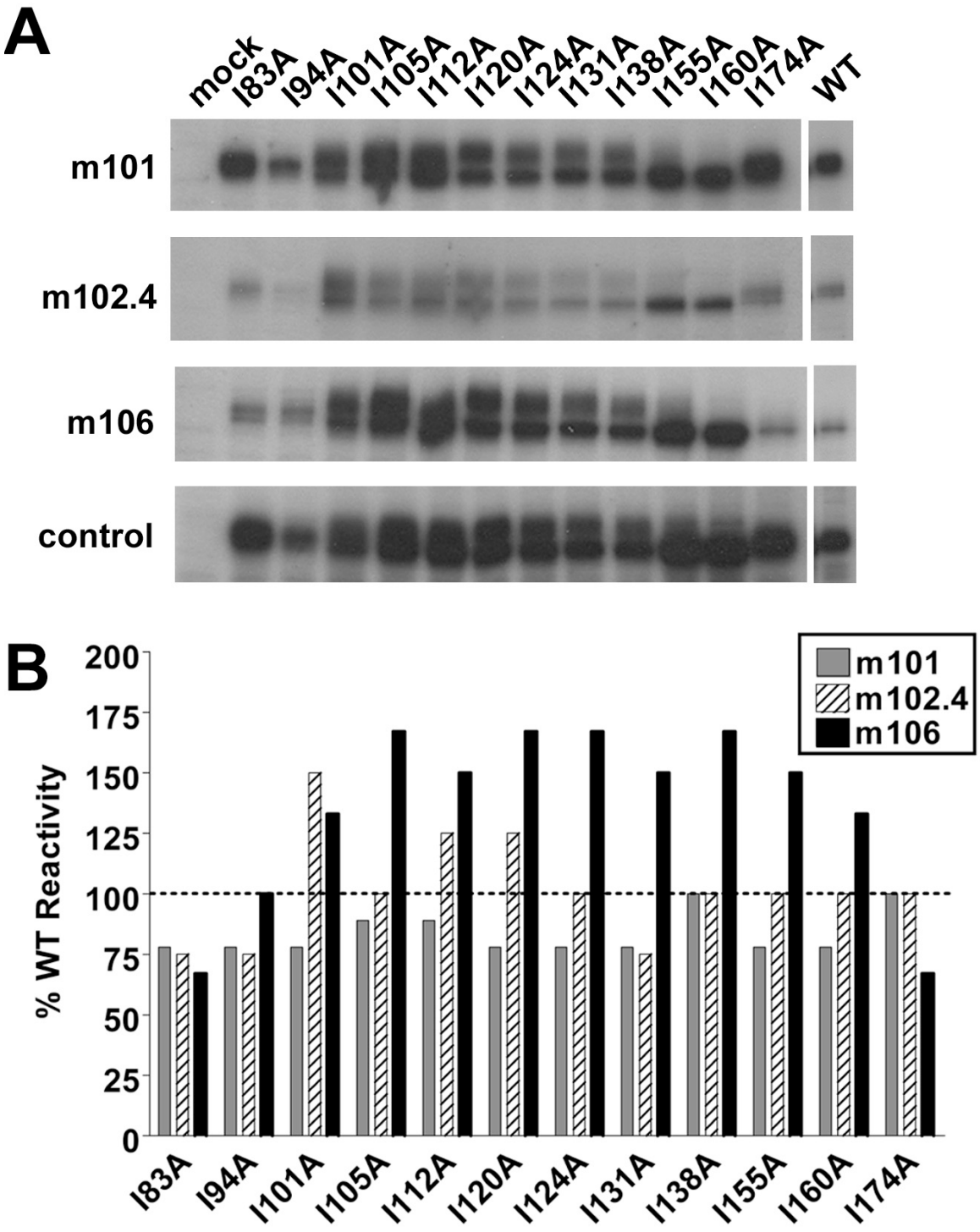

A
mock
I83A
I94A
I101A
I105A
I112A
I120A
I124A
I131A
I138A
I155A
I160A
I174A
WT
m101
m102.4
m106
control
B
m101
m102.4
m106
% WT Reactivity
200
175
150
125
100
75
50
25
0
I83A
I94A
I101A
I105A
I112A
I120A
I124A
I131A
I138A
I155A
I160A
I174A

Table 7: MAb reactivities to HeV G glycoprotein stalk mutants. Selected mAbs were assessed for their reactivities to the various alanine mutants versus WT HeV G in immunoprecipitation followed by SDS-PAGE and either western blotting or autoradiography. The results were quantitated via spot densitometry and the results are summarized here, where a "less than" symbol (<) means 50-75 % WT reactivity, "NC" means "virtually no change" or 76-125% WT reactivity, one "greater than" symbol (>) means 126-175% WT reactivity, and two "greater than" symbols (>>) means 176% WT reactivity or higher. "NT" means "not tested."

mutant G	8H4	m101	m102.4	hAH2.1	nAH22.4	m106	nAH23.4	nAH24.4
I83A	nc	nc	nc	nc	nt	-	-	nc
I94A	nc	nc	nc	nc	nt	nc	-	nc
I101A	nc	nc	+	nc	nt	+	nc	+
I105A	nc	nc	nc	++	++	+	+	++
I112A	nc	nc	nc	++	++	+	+	++
I120A	nc	nc	nc	+	nt	+	+	++
I124A	nc	nc	nc	++	nc	+	+	++
I131A	nc	nc	nc	+	++	+	nc	+
I138A	nc	nc	nc	+	nt	+	+	++
I155A	nc	nc	nc	+	nt	+	+	+
I160A	nc	nc	nc	+	nt	+	+	+
I174A	nc	nc	nc	nc	nt	+	-	nc

perhaps the boiled mutant G would still be better recognized by the antibody than the boiled WT G. Conversely, if the antibodies had an epitope that was partially conformation-dependent and partially linear, and if the antibodies were indeed recognizing a conformational difference between the mutants and the WT G, one would expect that pre-boiling the mutant and the WT G prior to immunoprecipitation with these antibodies would remove the difference between the amount of mutant G recognized versus the amount of WT G. Therefore, lysates containing various alanine mutants or WT G were pre-boiled for 5 minutes and then subjected to immunoprecipitation with one of the mAbs in question, nAH23.4. Under these conditions, the mutants and the WT G were all immunoprecipitated to similar levels; the apparent difference in nAH23.4's reactivity to the mutants versus the WT G had disappeared (data not shown). These findings supported the notion that the stalk mutations had not merely altered the antibodies' epitope(s) themselves, but had altered the conformation of G, and through 5 minutes of boiling the conformational difference was minimized. For these reasons and because the mutations causing this phenotype span a relatively large area of the protein, encompassing a stretch of at least 55 amino acids, we were then confident that the fusion-defective phenotype and the increased mAb reactivity observed in our stalk mutants was related to an altered conformation.

Discussion

Using a site-directed alanine-scanning mutagenesis strategy, the importance of the stalk domain of the HeV G attachment glycoprotein to its various structural and functional characteristics was examined by targeting a series of well-conserved isoleucine residues contained within this region. By altering these residues it was observed that this

region of the stalk domain was crucial to the fusion-promotion activity of the HeV G glycoprotein, and these results are complementary to the findings of other paramyxovirus attachment glycoproteins (26, 76, 79, 81). Notably, the mutation of any of the 12 isoleucines between positions 83 to 174 did not adversely affect the ability of G to interact with and coprecipitate the F glycoprotein nor did they affect the ability of G to engage ephrinB2 receptor, yet 9 of 12 mutants were completely defective in fusion-promotion activity.

The series of isoleucine residues mutated in the present study is homologous to a similar series of hydrophobic residues, many of which are isoleucine residues, within H and HN glycoproteins of several other paramyxoviruses. In particular, these residues in the NDV HN glycoprotein were hypothesized to form two heptad repeat-like structures and believed to be important for HN and F interaction (76). Additionally, during the preparation of this manuscript, a study by Corey et al. was published, in which mutations in the analogous region of the MeV H protein were mutated and found to decrease fusion-promotion activity without preventing F-interaction (22). The data presented here clearly indicate that this region within HeV G has a critically important role for conformation of the glycoprotein and in its fusion-promotion activity; however, like Corey et al., we found no correlation with a defect in the ability to interact with F. Further, although mutation of isoleucine stalk residues to alanine abrogated fusion-promotion activity in 9 of 12 cases, the G glycoprotein mutants were all cell-surface expressed, recognized by conformation-dependent mAbs and capable of binding the two viral receptors, ephrinB2 and ephrinB3. Although previous work with MeV has identified the minimal unit required for H dimerization to reside in the stalk domain (68),

we observed that our mutations in the stalk region of HeV G did not inhibit dimerization or tetramerization.

Unlike previous studies with stalk mutants of other paramyxoviruses, it was found that mutation of residues within the stalk domain appeared to result in an apparent differential N-glycosylation pattern (an altered doublet), but despite an extensive series of additional mutagenesis experiments we were unable to determine the precise nature of this change. The individual removal of all 8 potential N-glycosylation sites in some cases did reduce the levels of the higher molecular weight species but in no case completely prevented it and there was no evidence of any change in the fusion-promotion defective phenotype. In addition, although in previous studies that were unrelated to the mutagenesis of isoleucine stalk residues, increased N-glycosylation of other paramyxovirus fusion and attachment proteins has led to decreased fusion or failure to interact with the partner glycoprotein (1, 57, 85), there were no observed defects in either F or receptor interaction also making this an unlikely direct cause of the fusion-promotion defective phenotype. Additional studies of N-glycosylation site mutants in the context of WT G versus the I124A mutant could help elucidate the precise sites utilized in the WT G glycoprotein.

In addition to identifying and characterizing an important domain of G for its fusion-promotion activity it has perhaps more importantly been demonstrated here that receptor binding facilitates measurable conformational change in the HeV G glycoprotein as detectable by several conformation-dependent and partially conformation-independent (Western-blot reactive) mAbs. In most cases, efficient paramyxovirus-mediated membrane fusion requires the participation of both an attachment glycoprotein along with

an F glycoprotein, and F undergoes substantial measurable structural rearrangements upon "triggering" as it forms the 6-helix bundle structure concomitant with membrane merger (recently reviewed in (45)). For some paramyxoviruses, a physical interaction between F and its homologous attachment glycoprotein partner has been detected, including HeV, fostering the speculation that upon receptor binding, the attachment glycoprotein physically triggers F (reviewed in (8)), and the data in the present study are the first demonstration of receptor-induced conformational change in a paramyxovirus attachment glycoprotein that uses a protein receptor. The current study is also in agreement with the work of Takimoto et al. (78) which demonstrated alternative conformations in the NDV HN glycoprotein depending on whether it was or was not complexed with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (sialic acid), supporting the notion of receptor-induced conformational changes in an attachment glycoprotein which may facilitate the triggering of F fusogenic activity.

Moreover, it has also been demonstrated here that specific point mutations within the stalk domain of the G glycoprotein can cause similar conformational alterations in the protein in the absence of receptor that are normally detected only following receptor engagement; essentially these mutations substitute for receptor-induced alterations. We speculate that these G glycoprotein mutants are thus assuming a post-receptor binding conformation in the absence of receptor and thus are themselves already triggered upon expression and hence unable to fulfill their fusion-promoting activity when coexpressed with F even following subsequent receptor binding. This speculation suggested two important possibilities, the first being that the stalk domain of G plays an important role in the maintenance of conformation of G including regions within the globular head

domain. This idea is complementary to the findings of Wang et al., who found that the stalk domain of NDV HN was essential for neuraminidase activity, even though the neuraminidase active site is located in the globular head region (84). In addition, the findings of this study are also complementary to those of Deng et al., who observed that the stalk domain was important for maintenance of antigenic structures within the globular head domain as well as neuraminidase activity in HN (25). The second important implication of these studies is that through its governance of the conformation of G, the stalk domain may in effect regulate the timing of events in the fusion process, and that triggering events out of sequence can essentially block fusion, even after receptor engagement. Alternatively the mutants are "locked" in intermediate conformations that resemble receptor-induced conformations but unable to undergo further conformational changes required for fusion.

One can also speculate that because the human mAb 106 is a potent neutralizer (98) and binds better to some of the mutants, such mutants could be used as vaccine immunogens. In such immunogens the antibody epitope is better exposed and might lead to elicitation of m106-like antibodies.

We are presently conducting experiments to map the specific epitopes recognized by the mAbs used here, on the HeV and NiV G glycoproteins. Such knowledge will enable a better and more detailed understanding of the conformational rearrangements that G undergoes upon binding receptor as well as to help detail the multiple mechanisms of virus neutralization by antibodies. These results will also aid in understanding the process of paramyxovirus fusion in general and could potentially lead to the design of classes of antiviral drugs and vaccines.

Chapter 6: Discussion

Preface

HeV and NiV are recently emerged, novel paramyxoviruses. They are restricted to BSL-4 containment, can be isolated from natural reservoirs with relative ease, and have a distinctly broad host range. In addition, they are they are highly pathogenic in many animals and there are currently no approved therapeutics or vaccines. NiV, in particular, possesses several features which make it a significant biological threat, including person-to-person spread and high associated mortality upon infection (reviewed in (27)). These properties also make the henipaviruses potential bioterror agents and because natural henipavirus outbreaks continue to occur, it remains important that we study them further in hopes of developing antiviral measures and vaccines.

Although these and other characteristics set henipaviruses apart from other genera within the *Paramyxoviridae* family, they do possess some properties that are quite similar to other paramyxoviruses. For instance, the vast majority of paramyxovirus F glycoproteins cannot mediate fusion in the absence of attachment glycoprotein expression, and this is true for henipaviruses as well. In addition, amino acid sequence alignment of henipavirus envelope glycoproteins with those of other paramyxoviruses reveals spatial conservation of many important sequence motifs and structures, such as heptad repeats and cysteine bridges (39, 96). These and other important similarities should make the findings of the current study relatively generalizable to other paramyxovirus systems, at least within the subfamily *Paramyxovirinae*.

Accordingly, the goal of this study was to utilize henipaviruses as a model system for studying the mechanism of membrane fusion by paramyxoviruses. Several features

of henipavirus fusion have made these viruses ideal systems for use experimental approaches. For instance, the unusual robust nature of the henipavirus F/G interaction allowed coprecipitation of G with F and quantitation in the absence of any cross-linking reagents, which has historically not been possible in many other paramyxovirus systems. Secondly, the recent discovery of the host cellular proteins ephrinB2 and ephrinB3 as henipavirus receptors, and the presence of receptor-positive and receptor-negative cell lines, allowed the inclusion of these proteins in immunoprecipitation reactions in order to assess conformational changes induced by receptor binding. The observation of these receptor-induced conformational changes is an important finding, as such conformational changes in a paramyxovirus attachment protein have only been observed using the HN glycoprotein from NDV (reviewed in (8)). Thirdly, the robust interaction of G with the ephrinB2 and ephrinB3 receptors has made it possible to develop a coprecipitation assay for quantifying receptor binding by G glycoprotein point mutants. These and other features have made it possible not only to identify potential receptor binding residues of henipavirus G, but also to explore the mechanism of paramyxovirus fusion in the context of henipaviruses.

Discussion of the results with regard to the original hypotheses

The first specific aim of this work was to identify receptor binding residues in HeV G. The hypotheses were 1) that the receptor binding site of G is conformation-dependent and 2) that the globular head domain mediates interaction of G with receptor, with at least some important elements located in β-sheets 5 and 6, based on homology with MeV H. Using a site-directed mutagenesis approach, I successfully identified a series of residues in the globular head domain of HeV G which are critical for binding to the viral

receptors, ephrinB2 and B3. Specifically, these residues are D257, D260, G439, K443, G449, K465, and D468. Individual mutation of these residues to alanine resulted in decreased receptor binding and decreased fusion-promotion activity, as well as decreased reactivity of mAbs which were previously implicated in binding to the receptor binding domain (RBD).

Based on putative structures of the HeV G protein obtained through molecular modeling, these residues lie in β-sheets 1 and 4, which are two distinct, spatially-separated regions of the globular head domain. Based on these predictions, the residues in question either form a single discontinuous and conformation-dependent RBD, or two spatially separated RBD's. Presently, it is unclear which of these two possibilities is at play. Based on amino acid alignments with NDV HN, which has been co-crystallized with a receptor analog and shown to bind one receptor molecule on each of two sites, it appears that the RBD residues in β-sheets 1 and 4 of HeV G could be part of two separate RBD's. If that were the case, one might expect that a single point mutation at one of the sites, K443A for example, while perhaps abrogating binding at one site, would still allow binding at the second site. However, the K443A and K465A mutants each exhibit almost non-detectable receptor binding ability in the coprecipitation assay, and perhaps the simplest explanation is that there is indeed one discontinuous receptor binding site that is affected by each of these mutations– similar to the topology of the MeV H receptor binding site, which is composed of a series of residues that are not near each other in the primary amino acid sequence, but which are modeled to become proximal to each other and lie along the top of the head when the protein is folded into its native conformation

(54). Once a solved structure is available for henipavirus G, we may better understand the relative location of the β-sheet 1 residues with respect to the β-sheet 4 residues.

An original hypothesis of this work was that the RBD residues would be found in β-sheets 5 and 6, which is where the majority of MeV H SLAM-binding residues are located. In the present study, however, I identified residues in β-sheets 4 and 1. Interestingly, laboratory-adapted strains of MeV can utilize an alternate receptor for entry, CD46, and there is a cluster of CD46-binding residues in β-sheet 4 of the hemagglutinin glycoprotein from these strains (91). Therefore, based on the data presented here, it appears that although the original hypothesis that RBD residues would be found in β-sheets 5 and 6 was not the case, the rationale for that original hypothesis was correct, in that there is actually an important parallel with the structure and location of RBD residues between MeV H and HeV G. Additionally, although it is not presented as part of this work, I have recently obtained preliminary data using an additional panel of HeV and NiV G alanine mutants to expand our mutagenesis further around the globular head, and these data suggest that there are indeed RBD residues within β-sheet 5 of HeV and NiV G that are very analogous to the MeV H SLAM-binding residues (data not shown) and further confirm the importance of the β-sheet 4 region as well.

The second aim of this work was to explore the contribution of a specific series of hydrophobic residues between HeV F HRA and HRB to henipavirus fusion. Specifically, my hypothesis was that conserved isoleucines between HRA and HRB would be critical to HeV F-mediated membrane fusion. Each of the isoleucine, leucine, and valine residues from position 257-285 was mutated individually to alanine and the resulting mutants were tested for their fusion activity. While each mutation conveyed a decrease

in the fusion activity of F, the most dramatic effects were observed upon mutation of the isoleucine residues to alanine. Molecular modeling of F suggests these residues lie in a region of F which undergoes dramatic conformational rearrangement upon the switch from the pre-fusion to the post-fusion configuration. These results illustrate that indeed, the original hypothesis that this region would be critical to fusion was correct.

In a related aspect, the third aim of my project was to investigate the contribution of an isoleucine-rich domain in the stalk region of HeV G to the protein's various functional characteristics and roles in the virus infection process. The hypotheses were 1) that conserved isoleucine residues in the stalk domain of G are critical for fusion and 2) that conserved isoleucine residues in the stalk domain of G are not critical for receptor binding, oligomerization of G, or interaction with F. In order to accomplish this aim, 12 of 13 isoleucine residues in a conserved stalk domain motif of HeV G were mutated to alanine and the resulting G mutants were subjected to extensive biochemical characterization. The first observation was reminiscent of the HeV F data– that isoleucine to alanine mutations have drastic effects on fusion activity. This observation clearly addresses the first hypothesis– 9 of 12 mutations completely abrogated fusion; therefore the domain in question is critical to the fusion-promotion activity of G. Through biochemical characterization of the mutants, the second hypothesis was addressed: the stalk domain G mutations did not affect the receptor binding or F-interaction activities of G, nor did they affect the oligomeric characteristics of the glycoprotein. In light of what has been observed for other paramyxovirus attachment proteins, the fact that these mutations did not affect F-interaction or oligomerization was an important and somewhat unique finding in comparison to other paramyxovirus

systems. In fact, when these stalk mutants and the F mutants from Aim 2 were originally conceived, an alternative hypothesis that we were very interested in was that this isoleucine-rich domain in G and the hydrophobic motif we investigated in F would prove to be reciprocal G-F interaction domains, interacting through a leucine-zipper-like mechanism. Unfortunately, this did not turn out to be the case, and the G-F interaction domains remain to be elucidated.

In an attempt to understand how the stalk mutations facilitated such a drastic fusion defect, I further characterized the stalk mutants using monoclonal antibodies (mAbs), and here it became evident that the G mutants were in fact better recognized by mAbs which preferentially recognize receptor-bound G, causing us to conclude that the stalk mutants were assuming a post-receptor binding conformation in the absence of bound receptor.

Insights into the mechanism of fusion

The existing literature relating to membrane fusion and entry by paramyxoviruses highlights two central models for how G serves to trigger F. One model suggests that receptor binding by G induces conformational alterations of G, in the absence of changes in oligomeric status, and that these conformational changes are somehow transmitted to F and serve as the basis for triggering. The other model postulates that rather than receptor-induced conformational changes serving as the trigger for F-mediated fusion, receptor-induced oligomeric changes serve as the trigger (reviewed in (8)). Data obtained in the present study support the idea of receptor-induced conformational changes serving as the trigger for F-mediated fusion. Using purified human and murine mAbs, conformational alterations were detected in G after preincubation of G with soluble human ephrinB2 at physiological temperature. Furthermore, point mutants of G which were incapable of

fusion-promotion activity (and probably therefore most likely incapable of triggering F) were found to exist in what is thought to be a post-fusion or post-receptor binding conformation in the absence of receptor. This suggests that conformational changes do occur upon receptor binding, and that the timing of events is critical if membrane fusion is to proceed. The data also suggest that these conformational alterations induced in G upon receptor binding are subtle, as the above-mentioned mutants were found to be cell-surface expressed and recognized by every conformationally-dependent mAb with which they were tested. Importantly, these mutants that assume the putative post-receptor binding conformation were found to exist in oligomers very similar to that of WT G, which makes the model based on changes in oligomeric status seem even less likely, at least in the case of henipaviruses.

In addition to these two competing models of fusion, there has been historically some controversy in the literature regarding whether F and G are pre-associated prior to receptor binding or whether they associate only after receptor binding (58). In our laboratory, henipavirus F and G were found to coprecipitate when expressed in a nonpermissive cell line, HeLa-USU. Discovery of the receptors for henipaviruses and gene chip analysis of HeLa-USU cells as compared to permissive HeLa ATCC cells verified that HeLa-USU cells are receptor-negative (7). Nonetheless, when coexpressed in HeLa-USU cells (and in receptor-positive cells), F and G interact, as evidenced by coprecipitation. Additionally, in lysate-mixing experiments, when F and G are expressed singly in HeLa-USU cells and then mixed together in lysates under various conditions, they fail to coprecipitate (data not shown), suggesting that when F and G are expressed singly, either one or both glycoproteins assume(s) a conformation that is different than

when coexpressed with the partner glycoprotein. These data also suggest that F and G may arrive at the cell surface pre-associated, before receptor binding, which is in accordance with what is known about MeV envelope glycoproteins (69), but formal proof of this possibility remains to be obtained. Further evidence to support the notion of F and G pre-association was obtained in the current study: point mutants of G that were defective in receptor binding were found to still coprecipitate with F. In fact, upon quantitation, it was evident that most of them coprecipitated with F to levels twice that of WT G. These data suggest that receptor binding is not a prerequisite for F/G interaction.

The data obtained in the present study suggest this model for membrane fusion mediated by henipavirus envelope glycoproteins (refer back to **Fig. 1** in the Introduction): F and G are preassociated on the surface of cells prior to receptor binding. G binds to ephrinB2 or ephrinB3 on the host cell surface via residues in β-sheets 1 and 4 of G's globular head region, which induces a subtle conformational alteration. (This subtle alteration results in the better exposure of some mAb epitopes– m106, for example.) This conformational change in G is somehow transmitted to F, serving to trigger F to undergo conformational changes of its own. Membrane merger then proceeds via insertion of the fusion peptide into the target membrane and formation of the six-helix-bundle. The data also suggest that while it is the globular head region of G that is responsible for binding to receptor, the stalk region is equally important because it is the stalk which regulates conformational changes and the timing of events in fusion. Given the similarities that exist between henipavirus envelope glycoproteins and that of other paramyxoviruses, it is very likely that some or all of these findings may apply to the *Paramyxovirinae* subfamily overall.

Limitations and future directions

The work presented here adds much to our understanding of receptor binding and membrane fusion by paramyxoviruses; however, there are certain limitations to the current study. First and foremost, all of the work presented here was performed using recombinant expression of the viral glycoproteins in a vaccinia virus-based system to avoid the need for BSL-4 containment. Therefore, it is possible that in some aspects the experimental conditions did not reflect the molecular biology of a true henipavirus infection. Although it would be very interesting to validate these findings in the context of live virus experiments, there are some cases in which it may not be possible and some cases in which it may be unethical. An example of the latter case might be the receptor binding site studies; constructing a recombinant HeV with mutations in its attachment glycoprotein could potentially result in an unintended change in host range or pathogenicity– a type of experiment which many people would view as unacceptable for BSL-4 pathogens.

Additionally, all work presented here was performed using HeV envelope glycoproteins. Due to the high level of homology between HeV and NiV, and the ability of their envelope glycoproteins to function efficiently in heterotypic combination (12), we presume that most of the findings presented here would apply also to NiV, but most of these experiments have not been repeated using NiV glycoproteins. Additionally, we speculate that much of what we have learned here about henipavirus membrane fusion would also apply to other genera within the *Paramyxovirinae* subfamily, but it is possible that certain mechanistic differences may exist between G and H or HN.

Lastly, this work has been somewhat limited by lack of tools and reagents. By virtue of the fact that HeV and NiV are newly emergent viruses, they have not been studied for as many years as other viruses, such as Influenza and HIV. Therefore, fewer antibodies have been available for use in characterizing the envelope glycoproteins. Additionally, none of the mAbs that have become available during this study are completely mapped to specific epitopes. Having detailed information about the antibodies' epitopes would allow for more detailed and rigorous assessment of the effects caused by receptor binding to WT G, or the effects of specific point mutations constructed in F or G. Although during the course of this study we have developed both murine and human monoclonals to G, we do not yet have a single mAb specific for F. The lack of F-specific mAbs has greatly hampered our efforts to characterize F glycoprotein mutants.

In addition, there also has not yet been a solved structure for henipavirus G or for MeV H; the solved structures that do exist for paramyxovirus attachment proteins are those of various HN molecules. Although we have some computer-generated models of henipavirus G at our disposal, such models are obtained by threading henipavirus G residues onto already solved structures of HN molecules for other viruses. Such models are helpful, but hypothetical at best. When a solved structure does become available for henipavirus G, it will be very informative to locate on it the residues which have been demonstrated to affect receptor binding, as well as the residues implicated in receptor-induced conformational changes. It may then be possible to better understand the changes to the G glycoprotein once it binds its receptor, and how exactly G serves to

trigger F. Along these lines, it would be very interesting to compare the structure of a HeV G stalk mutant co-crystallized with receptor versus WT G thus co-crystallized.

Currently, our lab is working in collaboration with others to determine the crystal structures of henipavirus F and G glycoproteins, and to develop and characterize F-specific mAbs. Additionally, we are currently investigating another panel of HeV G glycoproteins mutants which extends our mutagenesis outward from the domains implicated as critical for receptor binding in Chapter 3. These mutants have alanine mutations of charged residues extending further around the globular head to β-sheet 5 and including several more strands in β-sheet 4, the loop connecting β-sheet 3 with 4, and a loop in β-sheet 1. Preliminary results obtained with these mutants already serve to further validate our findings that β-sheet 4 is a region of G that is critical to receptor binding, similar to the CD46-binding regions of MeV H. Additionally, this preliminary data suggests that there are RBD residues in β-sheet 5 of HeV G, similar to the SLAM-binding regions of MeV H. As a means of comparing the location of RBD residues in HeV to those of NiV G, a complementary panel of NiV G mutants is currently under construction. Preliminary results obtained with some of these NiV G mutants suggest that there are many similarities between HeV and NiV G in terms of how they bind receptor.

Unanswered questions

Although this work has generated much data and has made some significant contributions to our understanding of how paramyxoviruses mediate fusion, there remain unanswered questions about fusion. Examples of such questions include: What is the stoichiometry of the F-G interaction, meaning how many F molecules interact with how

many G molecules? Is it one F trimer per G tetramer, or a more complex relationship? Or, do F and G dissociate upon receptor binding?

In addition, there are some intriguing questions that have resulted from this work and which we do not yet have answers to. For instance, what is the reason for, or mechanism behind, some G mutants' hyperfusion phenotype, such as D470A and D564A? Another interesting and unexplained phenomenon observed in the course of this work is that some mutants of HeV G, in particular I83A and I94A, which do not have expression defects when expressed singly, appear to be somewhat down-regulated when coexpressed alongside HeV F. The reason for these mutants' lower expression when coexpressed with their partner glycoprotein is not understood, and somewhat hinders accurate quantitation of their ability to coprecipitate with F. However, although it was difficult to quantitate these mutants' coprecipitation accurately, they do still appear to complex with F, as evidenced by their lighter than WT, but still visible, banding pattern on IP-Western blots.

Contributions to the field of paramyxovirus fusion

The findings of my work, which have been summarized above, have made a significant contribution to our understanding of how paramyxoviruses mediate membrane fusion. For instance, the observation that HeV G mutants which are defective in receptor binding can still interact with and coprecipitate with HeV F, is important because it proves that receptor binding is not a prerequisite of F and G interaction– essentially laying to rest a fundamental controversy in the field. Another seminal contribution was the detection of receptor-induced conformational changes in G using monoclonal antibodies. This observation not only provides proof of a controversial step in the model

of paramyxovirus fusion but also explains the previously uncharacterized mechanism of neutralization by the human monoclonal, m106. Additionally, I identified several other antibodies which appear to recognize these conformational changes in G. To our knowledge, antibodies such as these have not yet been reported for another paramyxovirus attachment protein. In addition to clarifying the current model of paramyxovirus fusion, this work was also the first report of ephrinB3 receptor usage by HeV; this was previously only reported as an alternate receptor for NiV. This study was also the first to identify key residues of HeV G that are critical to its receptor binding ability.

Receptor binding and entry are the first steps in a productive infection, and therefore it is crucial that we continue to map the domains involved and ask mechanistic questions about the processes. In this way, we can add to our knowledge so that one day we may be able to rationally design antiviral drugs and protect against future outbreaks of these and other deadly emerging viruses. Although henipaviruses have only been recognized for the past 12 years or so, in this short time it has become evident that they have great potential to cause human deaths, as evidenced by the approximately 70% case fatality rate of the NiV outbreaks in Bangladesh. Additionally, they have the capacity to negatively affect economies that rely on livestock, like pigs, making them a potential agricultural bioweapon. One additional feature of NiV, in particular, makes these viruses even more of a threat, and that is the possibility of person-to-person spread. For these reasons, it is important to study the binding and entry process of henipaviruses so that perhaps we can find a way to intervene with a specific anti-henipaviral drug or vaccine. Finally, it appears that henipaviruses share a common mechanism of entry with other

Class I fusion viruses and perhaps in addition to designing specific antiviral substances, we may one day identify a common target in the process, such as membrane dimpling or curvature, and design an antiviral intervention that blocks entry of a wide variety of viruses that utilize this common pathway.

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